Chromosome Arrangement and Chromatin Structure in Mammalian Spermatogenesis

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B.Sc. (Hons.)

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ABSTRACT

The organization of the cell nucleus has been extensively studied for a long time before the development of *in situ* hybridization and chromosome painting in the last ten years produced direct evidence of nuclear organization. It is now known that chromosomes have defined non-overlapping territories that are non-randomly positioned within the nucleus, and which are inherited in somatic cells. The position of the chromosome depends on its replication timing and its content of active genes. Early replicating and active chromosomes are non-randomly positioned in the interior of the cell nucleus, and late replicating and inactive chromosomes at the periphery. Individual genes also have non-random positions, with active genes at the periphery of chromosome territories allowing transcription, and inactive genes positioned within the interior of the chromosome territory.

This raises the question of whether nuclear organization and specific chromatin structures persist between generations. A specific nuclear organization may be passed on to gametes, the zygote, and thence to helping to set up chromatin domains and expression patterns important for development. Previous studies of nuclear organization in vertebrate sperm have produced contradictory results with a random organization of chromosomes in amphibians and birds, but a non-random organization of chromosomes in several mammals.

To confirm my earlier finding of a non-random chromosome arrangement in sperm and to understand its generality and functional significance, I used chromosome painting to determine chromosome position in sperm of marsupials, monotremes and birds as well as of stages of meiosis. The chromatin structure of cells undergoing spermatogenesis was also studied in marsupials and eutherians to understand if specific arrangements and imprints could be passed on to the zygote that could influence zygotic gene expression or inactivation (eg. paternal X-inactivation in marsupials).

A non-random arrangement of chromosomes was observed in sperm of wombat (*Lasiorhinus latrifrons*). Comparison of the arrangement of homologous chromosomes between the two distantly related marsupials which diverged 50-60mya (the wombat and the dunnart) showed an identical arrangement suggesting that the non-random chromosome arrangement has an important function. Sperm of Platypus

(*Ornithorhynchus anatinus*) also had a non-random chromosome arrangement demonstrating that a non-random chromosome arrangement is a conserved feature of mammalian sperm. However, a random arrangement of chromosomes was observed in chicken sperm, which has the same fibrillar morphology and developmental sequence as in monotremes. This suggests that a non-random arrangement of chromosomes evolved in mammals for a mammal specific function.

A possible clue to this mammal specific function was my finding that the position of the X chromosome in sperm of all three mammalian groups was consistently at the region of the sperm that first makes contact with the egg. This meant that in all three mammalian groups, the X chromosome is the first part of the paternal genome to enter the egg. This specific position of the mammalian X chromosome may be important in setting up paternal X inactivation (thought to be ancestral).

This hypothesis assumes that the mammalian X is conserved in monotremes, as well as by eutherians and marsupials. To test the conservation of the monotreme X chromosome with the eutherian X chromosome, DNA from the flow-sorted platypus X chromosome was painted onto human metaphase spreads, producing one signal in male human metaphases and two signals in female human metaphases. The graded signal corresponded to the conserved long arm of the human X chromosome demonstrating conservation of the mammalian X chromosome over 170 million years. The conserved sequences on the X chromosome could be unique sequences, or conserved repetitive sequences (LINE-1) that could act as booster elements to promote X-inactivation along the mammalian X chromosome.

Chromatin organization can greatly influence gene expression and regulation. A specific chromatin structure may be passed onto the zygote imprinting specific regions of the paternal genome for expression or inactivation once it enters the zygote. The distribution of three different histones (H4ac, MacroH2A, and H2AZ) was observed in marsupial and mouse somatic cells and spermatogenesis, to understand how specific imprints may be passed on to the zygote. MacroH2A is associated with the inactive X in somatic and meiotic cells in eutherians. No macroH2A body was observed on the marsupial somatic inactive X chromosome, although it was observed on the marsupial sex vesicle. This suggests that the inactivation of the X chromosome in male meiosis may have been the conditions from which somatic cell inactivation evolved.

The distribution of a recently discovered histone variant, H2AZ, was observed in mouse spermatogenesis to try and understand its function. H2AZ was most

prominent in pachytene cells and round spermatids, where it localized to euchromatin rather than heterochromatin. It was absent from the inactive sex vesicle in pachytene cells. Overall regions of H2AZ enrichment lacked mH2A, and regions of mH2A lacked H2AZ. H2AZ was also present in stage 9-12 elongating spermatids and may play an important role in the nucleohistone to nucleoprotamine process.

This study has discovered that chromosome arrangement and chromatin structure is highly organized in mammalian spermatogenesis. This ordered process enables the inheritance of nuclear organization and imprints from the parent, setting up chromosome organization and gene expression in the zygote.

STATEMENT OF AUTHORSHIP

Except where reference is made in the text of this thesis, this thesis contains no material published elsewhere, or extracted in part or whole from a thesis by which I have qualified for, or been awarded another degree.

No other person's work has been used without due acknowledgement in the main text of this thesis.

This thesis has not been submitted for award of any other degree or diploma at any other tertiary institution.

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CHAPTER 1: INTRODUCTION

Within the nucleus chromosomes are positioned in relation to their gene content and activity. The chromatin structure is also dependent on gene activity, where inactive regions form a closed condensed chromatin state and active regions occur in an open chromatin state (reviewed by Cremer and Cremer, 2001). The nuclear organization is passed on from parental cell to daughter cell, allowing the inheritance of expression patterns between cells (Sun and Yokota, 1999). However, it is not understood if chromosome organization and expression patterns can be transmitted through meiosis from the parent to the zygote.

Little is understood about the nuclear organization within meiosis, during spermatogenesis and in the zygote. If a specific nuclear organization is transmitted to the zygote from the sperm, it may help in initiating expression patterns such as paternal X-inactivation. This study has aimed to determine whether a specific chromosome arrangement is present in mammalian sperm that may be used to set up chromosome domains and imprints within the zygote.

The chromatin structure was also compared between eutherian and marsupial meiosis, to try and understand if a specific imprint is developed on the paternal X chromosome predisposing it for X-inactivation. Several different imprints such as histone 4 acetylation, histone 3 methylation and different histone variants may imprint the sperm DNA influencing expression patterns within the zygote.

In this general introduction I will discuss chromosome organization. I will describe earlier work and its technical limitations, as well as the advances in this field due to the development of chromosome painting. Gene position in relation to chromosome position will also be discussed, and the correlation between gene expression and chromosome position, especially in the X chromosome. Chromosome organization at meiosis and in the zygote will also be reviewed. Since a chromatin structure could be retained from the sperm to the zygote, and might influence expression patterns, the components of chromatin will be discussed and their role in gene expression.

1.1 Chromosome structure and evolution

Before discussing nuclear organization, it is important to understand the composition and structure of the chromosome, and how chromosomes evolved over time. Many different techniques including staining, banding and now chromosome painting have been used to study nuclear organization. These techniques take advantage of the conserved chromosome features in helping to determine nuclear organization.

1.1.1 Chromosome structure

A chromosome is essentially one long DNA molecule, complexed and condensed with histone and non-histone protein. DNA in chromosomes is arranged as nucleosomes composed of DNA wrapped around histone octomers (section 1.6). Under the light microscope, various landmarks can be discerned - the centromere (a constricted region of DNA found on each chromosome that is essential for proper chromosome segregation at mitoisis and meiosis), chromosome ends (telomeres), nucleolus organizer regions (composed of rRNA genes, and heterochromatin that produces ribosomes). Euchromatin and heterochromatin may be distinguished by its staining properties (euchromatin is active DNA in an open chromatin state, whereas heterochromatin is inactive condensed DNA, in a condensed state) - two types of heterochromatin - constitutive (composed of repetitive sequences and present at all stages and tissues) and facultative (a region that undergoes changes in appearance and gene activity during development, e.g. inactive X).

The structure of a chromosome varies throughout the cell cycle. During interphase, individual chromosomes are not visible, but can be demonstrated to lie within a distinct domain. Different regions of euchromatin and heterochromatin are visible, and usually a single nucleolus at the centre of the cell. At prophase the chromatin begins to condense and the nucleoli disappear. After replication, interactions between separate nucleosomes, epigenetic modifications to particular histones and specific mitotic proteins, all lead to a condensed chromatin structure, known as the metaphase chromosome. After metaphase the chromosomes are moved to separate poles by spindles that are attached to centrosomes. At anaphase, microtubules extend from the centrosome connect to the kinetochore (a protein complex to which microtubules attach , allowing segregation of chromosomes), which is situated at the centromere of each chromosome, and separate the chromosomes to each pole during

anaphase. At telophase, the chromosomes move to separate poles and the cytoplasm and cell membrane divide through a process called cytokinesis producing two daughter cells (Campbell, 1993).

All of these features can and have been used to study chromosome organization during mitosis and interphase.

1.1.1.1 Staining and Banding

Early cytological experiments used staining techniques such as Feulgen or Giemsa staining which bind DNA and/or protein. In the 1970's techniques were developed to reveal light and dark banding patterns of chromosomes. This banding was first used in the comparison and identification of individual chromosomes. There are several banding techniques that help differentiate chromosomes from one another. Q-bands produced by staining chromosomes with quinacrine hydrochloride, which preferentially binds heterochromatin. G-bands are regions on the chromosome that are stained by Giemsa after proteolysis of the cell. G-bands have a high AT base content and represent inactive, late replicating DNA. R-bands are regions that are not stained with Giesma. These regions have a high GC base content and represent active, early replicating DNA, which is labeled with polysomal RNA (reviewed by Griffiths et al., 1996). C-banding is accomplished using Giemsa staining after denaturing chromosomes in sodium hydroxide (NaOH). This allows centromeric and other blocks of heterochromatin to be darkly stained.

1.1.1.2 Centromeres

Centromeres are loci on chromosomes that direct the attachment of spindle microtubules during division, allowing proper segregation and inheritance of chromosomes. Mammalian centromeres normally contain large amounts of satellite repeats, but these are not necessary for centromeric function (Lo et al., 2001). Centromeres are defined by specific proteins that bind to the centromere forming the kinetochore, to which the spindle joins (Smith, 2002). One important protein is centromeric protein A (CENP-A), which is believed to maintain the mammalian centromere (Henikoff et al., 2001). This is a histone 3 related protein that replaces histone 3 in centromere nucleosomes. CENP-A survives the protamine replacement step in spermatogenesis and allows the stable transmission of centromeres to the zygote. During mitosis other centromeric proteins bind to CENP-A producing the kinetochore allowing proper segregation of the chromatids (Henikoff et al., 2001).

Many different techniques have taken advantage of these and other centromeric features to study centromere positions in cells. For example, C-banding was used to study centromeric positioning in sperm of Urodeles (Dressler and Schmid, 1976) and chickens (Dressler and Schmid, 1976); in rat sperm a specific pericentromeric satellite repeat was used to observe centromeric positioning (Meyer-Ficca et al., 1998), and an antibody to CENP-A (centromeric specific protein and Histone 3 variant) was used to observe centromeric positioning in human sperm (Zalensky et al., 1995).

1.1.1.3 Telomeres

Telomeres are a combination of DNA and protein at the ends of chromosomes in eukaryotes, which provide the means to replicate and protect the ends of the linear chromosomes from degradation (reviewed by McEachern et al., 2000). Telomeres are normally long repeat sequences that have a single stranded overhang at the 3' end. In most vertebrates telomeres contain many tandem copies of a short DNA sequence (TTAGGG) (Meyne et al., 1989). This sequence is conserved in vertebrates including humans (Moyzis et al., 1988), because of its critical function as a protein binding site. *Drosophila melangaster* is exceptional in having a complex mosaic of large elements, primarily non-LTR retrotransposons that are added to the ends of chromosomes to counteract the gradual loss of sequences by incomplete replication. Most plant species share a conserved TTTAGGG telomeric sequence, but the species, *Aloe*, has the same telomeric sequence, TTAGGG, as humans (Weiss and Scherthan, 2002).

Telomeric shortening occurs during somatic growth because the requirement for a primer and the 5' to 3' polarity of DNA synthesis leaves one strand of DNA incompletely replicated. Yeast mutants that lack telomeric repeats ultimately undergo growth arrest (reviewed by McEachern et al., 2000). Telomeres and telomeric length are maintained by the enzyme telomerase, a protein complex that synthesizes more telomeric repeats. Telomerase contains an enzymatic core consisting of a reverse transcriptase (telomere reverse transcriptase, TERT) whose critical role is revealed by mutations that destroy telomerase function (Lingner et al., 1997). The enzymatic core also contains a telomeric RNA template, whose secondary structure is important for its activity and assembly with TERT (Gilley and Blackburn, 1999). The RNA sequence acts as a template from which telomeric DNA repeats are copied.

In some cases telomere fusions and recombination can maintain telomeric function in the absence of telomerase. In telomerase deleted yeast mutants, chromosomes fuse together at subtelomeric regions producing cells containing three

monocentric circular chromosomes (Nakamura et al., 1998). Loss of telomeres causes increased recombination with other telomeric regions, indicating that telomeric sequences can be maintained and expanded by recombination (Teng and Zakian, 1999).

Telomerase-independent telomere maintenance occurs in a small percentage of mammalian cancer cells and immortalized cell lines that lack telomerase. This process is referred as alternative lengthening telomerase (ALT) because telomeric lengths in these cells have increased (Bryan and Reddel, 1997). The process of ALT are as yet unknown, but it may involve recombination of telomeric sequences.

1.1.1.4 Nucleolus

The interphase nucleolus is organized around tandemly repeated genes that encode ribosomal proteins (rDNA) whose main purpose is the assembly of ribosomes. Two structures can be found within the nucleolus a dense fibrillar component that contains newly synthesized ribosomal RNA and a collection of proteins and a granular structure that is made up of ribosomal particles. During mitosis the nucleolus is disassembled and there is arrest of rDNA transcription. The rDNA genes are reactivated late in mitosis (Olson et al., 2000).

1.1.2 Vertebrate karyotypes

Each vertebrate group has its own characteristic karyotype that has variations between species.

1.1.2.1 Eutherian karyotype

Although the DNA amount is extremely uniform between mammal species, eutherian karyotypes are very variable. This contrasts with the karyotypes of birds and many reptiles that have virtually identical karyotypes containing a few large chromosomes and many tiny "microchromosomes" (Graves and Shetty, 2000).

Eutherians have highly variable karyotypes with large differences in chromosome numbers. For example, in the superorder Afrotheria the aardvark has a 2n=20 karyotype, whereas the African elephant has a 2n=56 karyotype (Yang et al., 2002). Chromosome morphology also is very different between species. For instance, all mouse chromosomes are acrocentric, whereas human chromosomes contain metacentric, submetacentric and acrocentric chromosomes. Eutherians generally have an XX female: XY male sex chromosome system or some variant of it.

1.1.2.2 Marsupial karyotype

The marsupial genome is about the same size, but is divided into a small number of very large chromosomes. The karyotypes range from 2n=10 to 2n=32 with most marsupials having 2n=14 or 2n=22 karyotype. The 2n=14 karyotype is virtually G-band homologous 2n=14 karyotype is represented in every marsupial superfamily in America as well as Australia, so is suggested to be ancestral (Rofe and Hayman, 1985). However, the presence of interstitial telomeres on these chromosomes in some species suggest that this karyotype arose by fusions from a yet more primitive 2n=22 karyotype (Svartman and Vianna-Morgante, 1999). Like eutherians, marsupials have an XX, XY sex chromosome system or a variant of it.

1.1.2.3 Monotremes karyotype

Monotreme karyotypes have a high chromosome number compared to most eutherians and marsupials. The platypus has a 2n=52 karyotype in both sexes, whereas the echidna has a 2n=63 (males) and 2n=64 (females) karyotype (Watson et al., 1992). This includes six large chromosomes and many small chromosomes. Platypus has an apparent XX, XY system, and echidna a X_1X_2Y (male) and X_1 X_1X_2 X_2 (female) system, although the participation of the sex chromosomes in a translocation chain of 8 (9) complicates the identification of sex chromosomes (Murtagh, 1977).

1.1.2.4 Bird karyotype

Bird karyotypes are rather uniform. Chickens have a 2n=78 karyotype that is nearly identical to the distantly related emu (Shetty et al., 1999). This includes 9 large macrochromosomes and many small microchromosomes. Birds have quite a different sex chromosome system from mammals, with ZZ males and ZW females.

1.1.3 Chromosome evolution

Throughout evolution, different chromosome rearrangements have taken place in different lineages to produce the wide variety of karyotypes and chromosome numbers. These include inversions, translocations, fusion and fission events. Inversions are intrachromosomal rearrangements when a chromosome region is inverted, producing a different G-banding pattern, and, if the centromere is included within the (Pericentric) inversion, a shift in centromere position. Translocations are the exchange between different chromosomes. Fusions are the joining together of separate chromosomes (often at terminal centromeres), to produce a smaller chromosome complement with larger chromosomes, eg marsupial chromosomes. Fissions refer to

the splitting of large chromosomes into smaller chromosomes to produce a larger complement of smaller chromosomes.

1.1.3.1 Vertebrate Phylogeny

Reptiles, birds and mammals shared a common ancestor 310 million years ago (mya). Three branches are recognized to have diverged after this time. Anupsidae includes turtles and tortoises; Diapsidae includes tuatara, snakes and lizards, crocodiles and alligators and birds. Birds diverged from the reptilian lineage 250mya (Benton, 1990) (figure 1.1). Mammals are the sole representatives of the third group Synapsidae.

Mammals are classified into two subclasses, the Prototheria and the Theria. Prototheria are represented by a single order (monotremes) containing the egg laying platypus and two echidna species. As yet, the phylogeny of mammals is under debate with two different hypotheses, the Theria hypothesis and the Marsupionta hypothesis.

The Theria hypothesis, comparisons of nuclear genes, fossil and anatomical evidence suggest that the subclass Theria (consisting of eutherians and marsupials) diverged from Prototheria about 170mya (Hope et al., 1990) (figure 1.1) and marsupials diverged from eutherians about 130 mya.

The Marsupionta hypothesis, suggests that monotremes and marsupials are more closely related than eutherians. This theory was established through sequencing of 12 mitochondrial DNA (mtDNA) genes (Janke et al., 1996). Phylogenetic analysis of the 18s rRNA gene and the mitochondrial genomes of wombat (*Vombatus ursinus*) and the spiny anteater (*Tachyglossus aculeatus*) suggested that Marsupionta (monotremes and marsupials) diverged from eutherians 130 mya and monotremes diverged from marsupials 72 mya (Janke et al., 2002).

Marsupials irradiated from North America into South America earlier than 65 mya. Marsupials then moved through Antarctica, and into Australia when both continents were connected. The separation of Australian marsupials from South American marsupials occurred 84-38 mya and the oldest Australian marsupial fossils are dated at 55mya (reviewed by Graves and Westerman, 2002).

There are seven orders of marsupials contain 20 families, with two of these orders South American. The order dasyuridae (containing dunnarts) diverged from the order Diprotodontia (containing wombats and the tammar wallaby) 50-60mya (figure

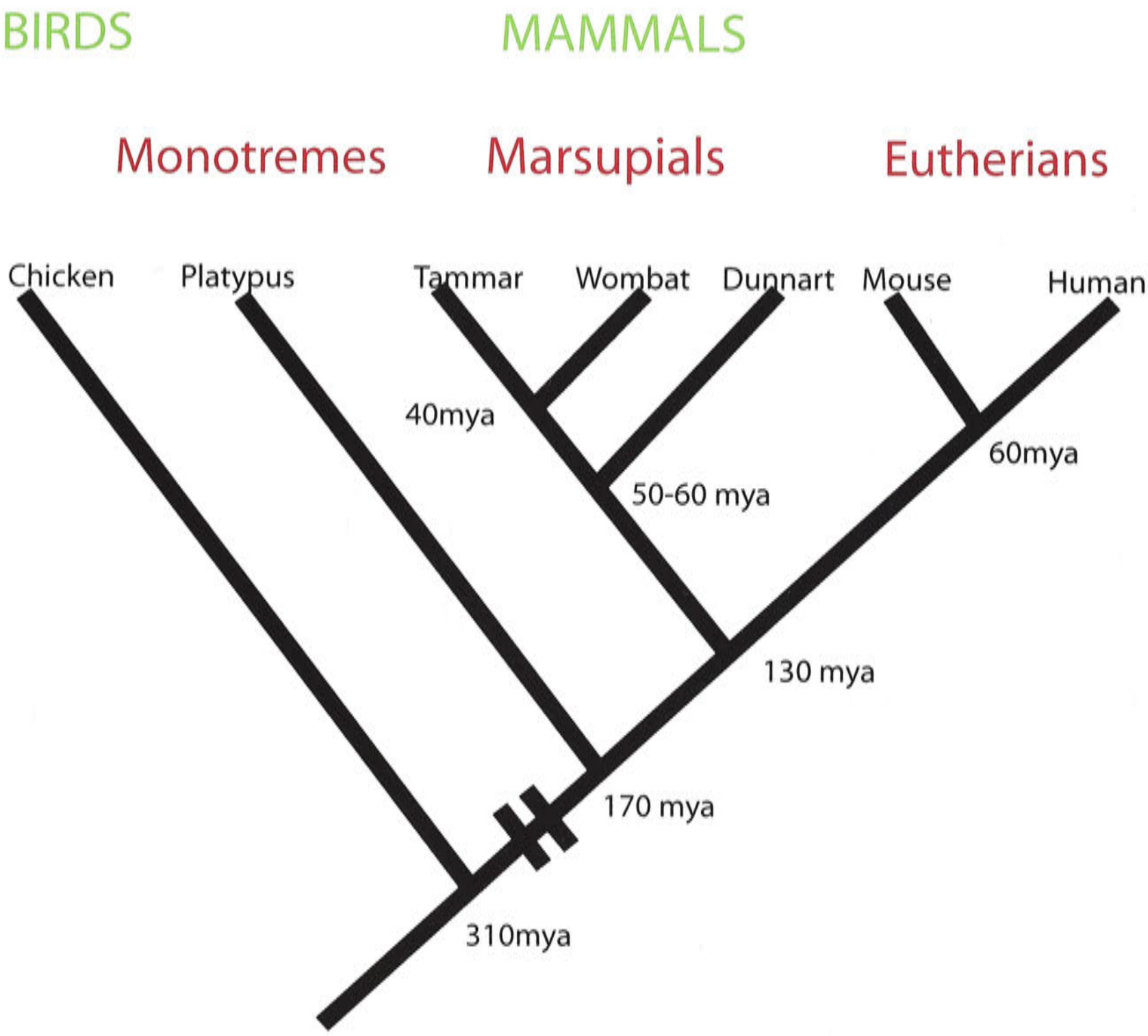


Figure 1.1: Vertebrate phylogeny. The above tree illustrates relationships and dates of divergence of species relevent to this study.

1.1). The last divergence in marsupials relevant to this study is the divergence of the suborders Vombatiformes (wombat) and the suborder Macropodiformes (tammar wallaby) occurred 40mya (Kirsch et al., 1997) (figure 1.1).

1.1.3.2 Comparisons using chromosome painting

The homologies of chromosomes and chromosome regions may be directly compared using *in situ* hybridization with DNA from isolated chromosomes. Flow-sorted chromosomes provide these chromosomes - specific probes that can be used to study chromosome evolution. Chromosomes can be distinguished by their size and base content using The FACS (fluorescence-activated chromosome sorter) (Ferguson-Smith, 1997). Peaks that represent a single chromosome can be distinguished and can be physically separated.

The small amount of DNA contained in flow-sorted chromosomes can be amplified by a degenerate oligonucleotide PCR, and labeled by incorporation of biotin and/or digoxigenin. (Telenius et al., 1992). These labeled chromosome-specific "paints" can then be hybridized to the homologous species to check specificity, then to related species to observe karyotypic evolution (Ferguson-Smith, 1997, Ferguson-Smith et al., 1998). Chromosome painting allows the detection of homology of chromosomes or chromosome regions between different species. The success of the painting greatly depends on the sequence of homology the two species share. In closely related species (such as primates) there is good cross-species homology, but cross - species painting between more distantly related species (such as humans to mouse) is poor. The successful hybridization of the tammar X chromosome to the human X chromosome was hailed as a breakthrough, considering the 130 million years of divergence between the two species (Glas et al., 1999).

1.1.3.3 Chromosome evolution in mammals

Chromosome painting has been used to study karyotypic evolution in mammals. The aim of these experiments is to understand the pattern of rearrangements that produced the present karyotypes and to know the ancestral karyotype. The use of chromosome paints has helped in defining conservation of whole chromosomes and large chromosomal blocks between eutherian species. Some human chromosomes such as human chromosome 13 (HSA13), HSA17, HSA20 and HSAX, have had no interchromosomal rearrangements in any of the eutherian species studied (reviewed by Chowdhary et al., 1998). Human chromosomes HSA9, HSA2, HSA4, HSA6, HSA16

and HSA19 all have large segments conserved throughout the eutherian karyotypes. For example, human chromosome 9 has been conserved as a single chromosome in carnivores and as a single block in pig and cattle. The conservation of this chromosomal block means that it probably corresponds to an ancestral mammalian chromosome. The form that an ancestral eutherian karyotype might have taken is still under debate, and proposals include 2n=14 to 2n=48 karyotypes (reviewed by Chowdhary et al., 1998) with the latest proposal being 2n=44 (Yang et al., 2002).

1.1.3.4 Chromosome evolution in marsupials

There has been longstanding debate over whether the marsupial ancestral karyotype was 2n=14 or 2n=22. Two theories have been proposed, one, that fissions occurred to a 2n=14 ancestral marsupial karyotype (Hayman and Martin, 1974, Rofe and Hayman, 1985), or two, that fusions occurred to a 2n=22 ancestral marsupial karyotype (Sharman, 1961, Svartman and Vianna-Morgante, 1998).

Karyotypes of numerous 2n=14 species show widespread occurrence of 3 large metacentric or submetacentric chromosomes, 1 medium sized pair near metacentric chromosome, two smaller pairs of submetacentric chromosomes and small X and Y chromosomes. The occurrence of this conserved karyotype in every marsupial superfamily, including Australian and American families, suggested that the 2n=14 karyotype was the ancestral marsupial karyotype (Hayman and Martin, 1974). Further evidence for this theory came from the virtually identical G-banded metaphase chromosomes between 6 Australian 2n=14 marsupials (*Vombatus ursinus, Cercartetus concinnus, Acrobates pygmaeus, Isoodon obesulus, Permeles nasuta, Ningaui* sp.) and one American 2n=14 marsupial (*Caluromys lanatus*) (Rofe and Hayman, 1985).

The second proposes that the ancestral marsupial karyotype derived from a didelphid like ancestor with 2n=22. In this scenario, evolution in marsupials was accompanied by a general reduction in chromosome number (Sharman, 1961). This theory was widely disregarded due to the results of later studies (Rofe and Hayman, 1985). However, recently the fusion theory has again been proposed. G-banding of the South American *Marmosops incanus* and the occurrence of interstitial telomeric sequences suggested that the 2n=14 karyotype was produced by a series of Robertsonian fusions from a 2n=22 ancestor (Svartman and Vianna-Morgante, 1998). It is likely, therefore, that the ancestor of Australian and South American marsupials with a 2n=14 karyotype derived from an older S.American marsupial lineage with 2n=22.

Chromosome evolution has been extensively studied using chromosome painting. Tammar wallaby chromosome-specific paints have been hybridized to dunnart and wombat (2n=14) metaphase spreads (De Leo et al., 1999). The tammar wallaby chromosome paints hybridized in the same or similar patterns to corresponding chromosomes in both species. Tammar wallaby chromosomes 3 and 5, hybridized in one block to dunnart and wombat chromosomes 5 and 3 respectively. Chromosome 2 of the tammar wallaby hybridized to the whole chromosome 4 in both the wombat and the dunnart. Chromosome 1 in the dunnart and the wombat was the only chromosome to involve three different tammar wallaby blocks (figure 1.2). The common ancestor underwent two different inversions and two fissions to create the three blocks found in the tammar wallaby (De Leo et al., 1999). Thus wombat and dunnart chromosomes seemed to be entirely conserved apart from intrachromosomal rearrangements (inversions) on chromosomes 1, 2 and 6 (De Leo et al., 1999). The conservation of karyotypes between dunnart and the wombat implies that the ancestral macropodid karyotype (2n=22) derived from the Australian marsupial ancestral 2n=14 karyotype.

1.1.3.5 Chromosome evolution in birds

In birds and reptiles, chicken chromosome-specific paints have been used to establish chromosome homologies to other birds and even reptiles. Conservation between all chicken and emu macrochromosomes was demonstrated, apart from chicken chromosome 4, which hybridized to emu chromosome 4 and a pair of microchromosomes. Chicken macrochromosome paints also hybridized to whole turtle macrochromosomes, with the exception (again) of chromosome 4, which hybridized to turtle chromosome 4 and the short arm of a small submetacentric (Graves and Shetty, 2000). The demonstration that an emu microchromosome corresponds to two different regions of larger chromosomes in chicken and turtle suggested that the ancestral karyotype might have included a microchromosome that fused with different elements to form larger chromosomes (Shetty et al., 1999, Graves and Shetty, 2000).

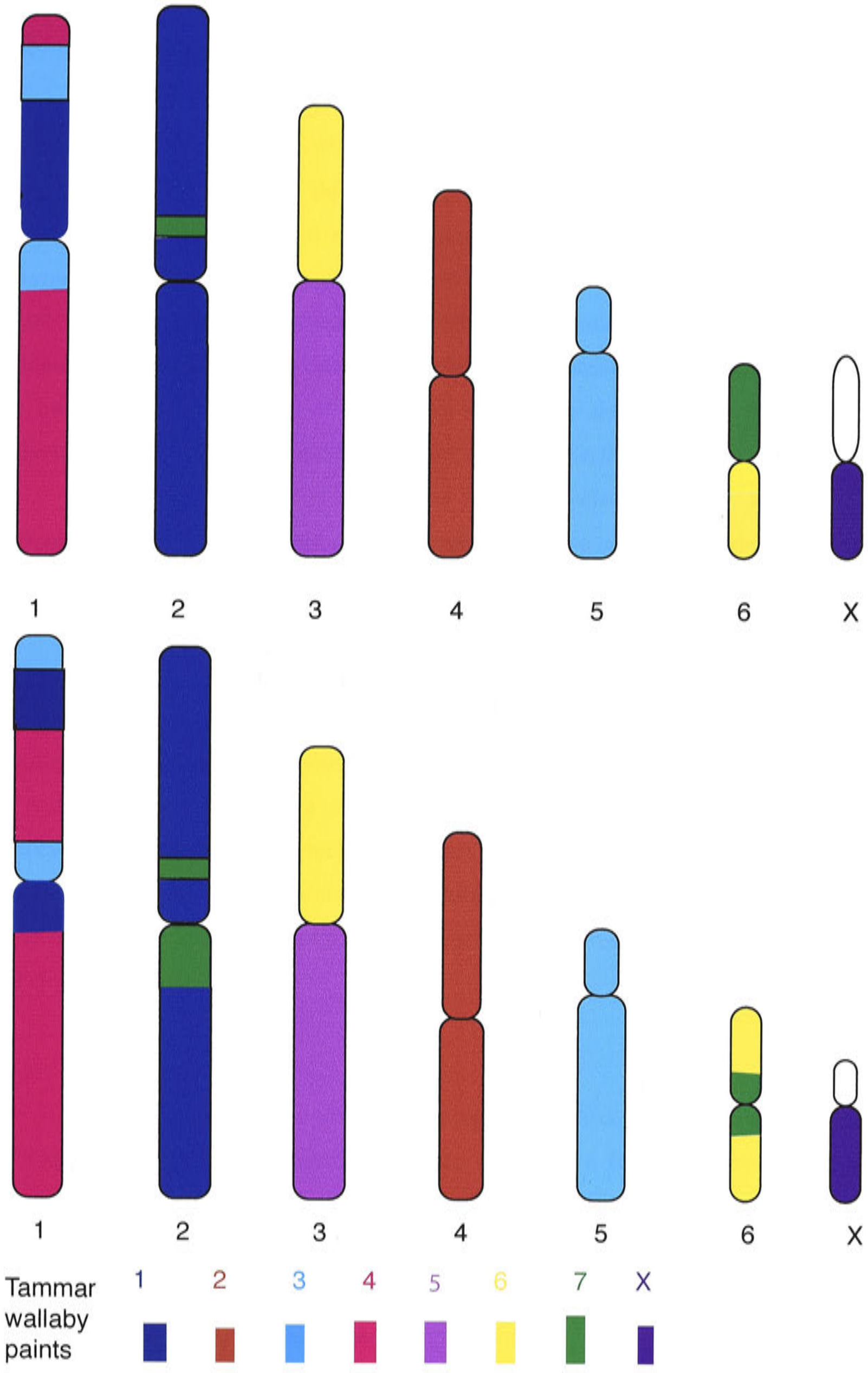


Figure 1.2: Conserved karyotypes (2n=14) of the wombat (a) and dunnart (b). Chromosomes are identical with the exception of intrachromosomal rearrangements (inversions) in chromosomes 1, 2, 6 (modified from De Leo *et al*, 1999).

1.2 Nuclear organization.

The term nuclear organization, describes the organization of DNA and associated proteins within the cell nucleus. In the interphase nucleus chromosomes are found in their own discreet domains with no overlapping between chromosomes. Nuclear organization also describes the chromatin state within a cell nucleus.
Chromatin (DNA and DNA packaging proteins, eg. histones) comes in two different forms, heterochromatin and euchromatin, with heterochromatin representing inactive condensed DNA and euchromatin representing uncondensed active DNA. Within the cell nucleus different regions of replication timing can also be observed. However, without scientific methods little of the nuclear organization described can be observed.

The nuclear organization of a cell is also cell cycle dependent. Although it is difficult to interpret nuclear organization within interphase cells, the condensation of the DNA at metaphase allows the recognition of each separate chromosome. As described previously different regions of the chromosome can also be recognized at this stage (section 1.1.1).

Early work on nuclear organization in somatic cell types used indirect techniques and produced inconsistent results. The life cycle of cells, including phases of replication have made the study of chromosome organization more complex. However, new equipment and techniques have led to major leaps in our understanding of the arrangement of chromosomes and genes in a cell. The major revolution in the study of nuclear organization was the development of fluorescence *in situ* hybridization and chromosome painting.

1.2.1 The pre-painting era

The early research into chromosome organization especially using the limited techniques, equipment and knowledge of the time was a frustrating part of science. However, ingenious methods were devised to infer the arrangement of chromosomes in the interphase nuclei of animal and plant cells. Many of these earlier studies used mitotic cells, as chromosome position is easiest to discern during metaphase when chromosomes condense and individual chromosomes can be recognized.

Early work provided evidence for pairing of homologous chromosomes in interphase somatic cells (reviewed by Comings, 1980). Unfortunately not all studies

agreed with these findings. The suggestion of homologous pairing was based on evidence of centromeric associations of homologous chromosomes in root tips. This was believed to demonstrate universal somatic association of homologues in somatic cells and mitotic cells. However, through the use of glutaraldehyde fixation and serial sectioning it was later shown that homologues were not associated, and the earlier results were a by-product of the methods used (reviewed by Heslop-Harrison and Bennett, 1990). The finding of close associations of homologous chromosomes can be attributed to the nucleolus. 5sDNA genes are associated with the nucleolus, and therefore chromosome pairs carrying these genes will be positioned close to each other, providing the observation of a close association of homologous (Comings, 1980).

The inconsistencies in non-random arrangements of chromosomes in mitotic spreads may be due to the techniques used to prepare the cells. Chromosome organization was visualized in squashed preparations treated with colchicine to disrupt spindles and hypotonic to swell the cells. Early research showed that colchicine and Colcemid as well as hypotonic treatments, destroyed chromosome orientation in the cell (Cohen et al., 1972, Heneen and Nichols, 1972). Such techniques cannot provide an accurate representation of chromosome position in the living three dimensional cell. As stated by Bennett (1982), "studying squashed preparations of mitotic cells is like trying to reconstruct an egg that has been thrown against a door".

Such inconsistencies in results were observed between Chinese hamster testicular mitotic cells (germcell) and Chinese hamster fibroblaast mitotic cells. In testicular mitotic cells, chromosomes were non-randomly distributed in four groups, with chromosomes 9, 10 and 11 in close association, 7 and 8 in close association, 1 and 2 in close association and chromosomes 3, 4, 5 and 6 in close association. Within these groups chromosome positions were random (Juricek, 1975), although the smaller chromosomes were preferentially found within the centre of the metaphase plate. In cultured fibroblast mitotic cells there was no evidence for any non-random chromosome organization (Hens, 1976).

In some hybrid plants, chromosomes at the periphery of the cell nucleus are preferentially lost from the cell (Bennett, 1984), whereas in *Hordeum x Secale* hybrids, the two parental genomes stay separate within the nucleus (Bennett, 1982). Treatment with a spindle poison disrupted genome separation and removal of the poison permitted separation to occur again (reviewed by Heslop-Harrison and Bennett, 1990). This suggested that microtubules play a role in maintaining chromosome positioning within a

cell. It was not until the use of serial sections and electron microscopy that a better understanding of chromosome organization was developed.

In serially sectioned human fibroblast cells the small chromosomes were found to be more central in the metaphases and the larger chromosomes were more peripheral (Mosgoller et al., 1991). In another study of human mitotic chromosome position, the morphology and size of chromosomes was used to discern a non-random position of smaller chromosomes towards the centre of the metaphase spread (Lichter et al., 1988). The position of chromosomes at metaphase generally reflects the order and position of chromosomes in interphase cells (Leitch et al., 1990). In animal cell hybrids produced by fusing hamster and human cells, the hamster chromosomes were on the outside and the human chromosomes on the inside of the metaphase plate.

Although the organization of interphase cells was studied from as early as the 1940's, the observations were always indirect (reviewed by Haaf, 1991 #120]). The demonstration that the inactive X chromosome in eutherian female cells formed the "Barr body" (sex chromatin) at the periphery of the interphase nucleus (Barr and Bertram, 1949) was one of the first, and best, direct demonstrations of a non-random position of chromosomes in cells, and the first demonstration that the less active chromosomes lie towards the periphery of the nucleus (Croft et al., 1999).

One of many different indirect techniques used to study chromosome organization in an interphase cell was irradiation, of cells to produce double stranded breaks in the DNA. If chromosomes were randomly positioned, there would be an equal probability of the broken DNA reuniting with broken DNA from any chromosome. However, the aberrations were distributed non-randomly, with a greater frequency in exchange between centromeric and heterochromatic DNA, providing indirect evidence of a non-random chromosome organization in interphase cells (reviewed by Comings, 1968).

Even as late as the early 1980's, there was no direct evidence of a high level of chromosome organization (reviewed by Comings, 1980). The big breakthrough in the study of chromosome arrangement was the development of *in situ* hybridization, which uses hybridization of specific probe DNA to complementary sequences in the genome to identify a particular sequence within the nucleus. Initially, probes consisted of repetitive sequences (eg. Centromeric, heterochromatin, Alu sequences, telomeres), but with the refining of techniques, chromosome-specific libraries and single gene probes were used to look at chromosome, and gene positions within a cell nucleus. In

conjunction with 3D reconstruction, fluorescence microscopy, serial sectioning and electron microscopy, fluorescence-labeled DNA probes have become powerful tools in observing chromosome organization.

1.2.2 Advent of FISH and chromosome paints

Use of fluorescence *in situ* hybridization and chromosome paints lead to major advances in the study of nuclear organization, enabling more specific studies of chromosomal arrangement in different cell types and phases. Multicolour FISH allows the recognition of multiple chromosomes at the one time and allows the relationship and position of two different chromosomes to be compared. These techniques allow specific regions of DNA, whether it is repetitive or whole chromosomes, to be visualized. This is an advantage for interphase cells, where separate chromosomes cannot be visualized without the use of chromosome paints.

1.2.2.1 In situ hybridization and chromosome painting at mitosis

The use of chromosome paints allowed more specific and accurate analysis of chromosome positions at mitosis. For instance, when human chromosome paints were used to investigate the angular separation of chromosomes on the wheel-like rosette (chromosomes distributed in a circular pattern) of a human metaphase homologous chromosomes were never found within 90° of each other. If chromosome positions were random 50% of homologues would be expected to be within 90° of each other. This suggests non-random positioning of chromosomes at metaphase. The degree of separation between homologous chromosomes 1, 7, 8, 9, 16 and the X ranged from 140° and 160° apart (Nagele et al., 1995).

Using chromosome painting, chromosomes were shown to have a consistent spatial order within the rosette. For instance, chromosomes 16 and X were adjacent to each other in prometaphase rosettes of human diploid fibroblasts, as well as in interphase cells.

Not all the literature agrees with the conclusion of a non-random position of chromosomes at mitosis. For instance, in another study of mitotic chromosome positions in cultured human lymphocytes and fibroblasts the X and Y chromosomes had variable positions on the metaphase rosette (Allison and Nestor, 1999). There may be many different reasons for the inconsistent results. Different cell lines were used between the two studies and therefore, chromosome organization may differ between

cell types as Nagele (1995) used human fibroblast cells and HeLa cells, whereas Allison (1999) used cultured human lymphocytes MRC-5 and CCD-34Lu. Both studies used similar fixing techniques and therefore this did not create the differences in results. Allison (1999) prepared some of these cell types by dropping the cell suspension from a height of 10cm. Such a technique may disrupt the nuclear organization present in these particular cells.

Unfortunately it is impossible to determine which paper is more accurate, and again highlights the problem in studying nuclear organization. There seems to be no reasonable explanation as to why the results between these two papers are so contradictory.

The similarity of order and relationships of chromosomes in metaphase spreads and interphase cells suggests that there is some mechanism to maintain chromosomes in specific positions between cell generations. It has even been suggested that chromosomes are permanently attached physically through centromeric interconnections that line up in a 'string of pearls' at G_2 and form a hub at prometaphase (Nagele et al., 1995).

1.2.2.2 In situ hybridization and chromosome painting in interphase cells

Since the late 80's direct observation by chromosome painting has greatly clarified chromosome positioning in interphase cells. It is now obvious that chromosomes have a high level of nuclear organization within a cell (reviewed by Cremer and Cremer, 2001), that chromosomes have discreet boundaries within a cell nucleus (Lichter et al., 1988), that a chromosome order is established early in the cell cycle (Croft et al., 1999), is cell type dependent (Manuelidis and Borden, 1988), and that the order seen in a cell is passed onto the daughter cells (Sun and Yokota, 1999).

In one of the earlier studies using centromeric probes, computer reconstructions of serial sections displayed a cell specific non-random pattern (Manuelidis, 1984). Centromeric positions were compared in several highly differentiated central nervous system cells, which offer the advantage that they do not replicate, so chromosomes do not move in relation to the cell cycle. The study found that Purkinje neurons had two large centromeric clusters in the centre of the nucleus, whereas granule neurons had 4 or 5 large centromeric clusters around the periphery of the cell nucleus (Manuelidis, 1984). Centromeric probes have shown that human chromosomes 4, 6, 10 and 17 have the same position in daughter cells (Sun and Yokota, 1999). This showed evidence of

chromosomes being placed in a particular position from which they never move once the order has been set.

Chromosome specific libraries were subsequently used to mark the positions of whole chromosomes or chromosome arms within a cell (Manuelidis and Borden, 1988). These chromosome paints gave a better indication of chromosome behaviour within a cell than did probes to specific regions such as centromeres, telomeres, and heterochromatin. For example, centromeres constitute only small parts of a chromosome; so interior positioning of the centromere gives no indication of the positions of the arms, and therefore position of gene rich areas of the chromosome. Also most centromeric probes do not differentiate between particular chromosomes. Chromosome painting and chromosome specific libraries allowed the position of a whole chromosome to be observed, as well as its relationship with other chromosomes which (as will be discussed in 1.4.4) may be important in normal functioning and disease.

Chromosome painting showed for the first time that chromosomes occupy their own discreet compartments rather than the DNA being intermingled in interphase nuclei (Lichter et al., 1988). The chromosome compartment itself is also organized into regions of early and late replicating DNA.

In the last decade, chromosome painting has been used to document the arrangement of chromosomes in many different cell types. For instance, in quiescent human fibroblast cells, chromosomes 7 and 8 were found at the periphery, whereas chromosome 16 had a more internal position (Nagele et al., 1999). Interestingly this same article reported that the X chromosome has a more random position.

Chromosome positions and relationships may also lead to rearrangements important in diseases. For instance, chromosome 9 and chromosome 22 usually lie close to each other at the centre of the nucleus in G_0 phase lymphocytes (Kozubek et al., 1999). This may ensure that they undergo exchanges at a higher than expected frequency, explaining the high rate of induction of t(9;22) –positive leukemias in the human population. A recurrent primary abnormality in chronic myleiod leukaemia is a translocation involving the *ABL* (chromosome 9) and *BCR* genes (chromosome 22). These chromosomes are in close proximity to each other at interphase and are frequently involved in exchange aberrations after neutron irradiation. In contrast, chromosome 8, which has a non-random position towards the periphery of the nucleus (Kozubek et al., 1999), was infrequently involved in aberrations with chromosome 22.

It is now clear that chromosomes have non-random positions within a cell, but it is still not clear what factors influence the arrangement of chromosomes in the interphase cell and how these non-random chromosome arrangements are inherited through meiosis.

1.3 Factors involved in nuclear organization

A variety of hypotheses have been put forward to account for the non-random chromosome arrangement in somatic cells. Factors proposed to determine chromosome arrangement include chromosome size (Habermann et al., 2001, Mosgoller et al., 1991, Sun and Yokota, 1999), asynchronous replication (Li et al., 1998) and gene content and expression (Croft et al., 1999).

1.3.1 Size dependent chromosome organization

As discussed in section 1.2.1, there are examples in which smaller chromosomes are non-randomly positioned in the interior, and larger chromosomes at the periphery of the metaphase spread (Mosgoller et al., 1991), as well as examples of size dependent chromosome organization in interphase cells (Sun and Yokota, 1999).

In human cells, chromosome-specific subtelomeric probes were used to show that large chromosomes were more peripherally located, and smaller chromosomes were positioned towards the centre of the nucleus (Sun and Yokota, 1999).

A relationship between chromosome position and chromosome size has also been observed in chickens, which have extreme size differences between nine large macrochromosomes and many smaller microchromosomes. Chicken chromosome paints were used to study the position of macro- and microchromosomes at metaphase. Microchromosomes were found in the centre of the metaphase spread surrounded by the macrochromosomes (Habermann et al., 2001). Chicken chromosomes also had a non-random radial position within the interphase cell nucleus, with microchromosomes in the interior, and macrochromosomes at the periphery. This organization was seen in different cell types, so may represent a general organization found in all chicken somatic cells. However, size of chicken chromosomes may be only indirectly related to chromosome position, since microchromosomes are early replicating and gene rich, both factors which have been implicated in chromosomal organization [Croft, 1999 #71; (Ferreira et al., 1997).

1.3.2 Relationship of chromosome arrangement to replication timing

There is evidence that early and late replicating chromosome regions are found in distinct nuclear territories. Pulse incorporation of halogenated deoxynucleotides such as bromodeoxyuridine (BrdU) was used to observe the spatial position of chromatin replicating at different stages of the cell cycle. Early replicating DNA was more interior and late replicating DNA was more peripheral in the nucleus (Ferreira et al., 1997).

The banding patterns were observed by using halogenated deoxynucleosides corresponded to R and G-banding. G-bands correspond to gene poor areas and are late replicating, whereas R bands are gene rich and early replicating. In telophase/early G_1 stage the late replicating regions were at the periphery of the cell nucleus and the early replicating regions were towards the centre of the nucleus. At metaphase these previously separated bands form an intercalated pattern on the mitotic chromosomes. This consolidation of late and early replicating regions in interphase cells reflects different areas of differential gene expression.

Early and late replicating parts of the chromosome are also separated within single chromosome territories in interphase cells. For example, Chromosome 12 can be divided into 5 distinct replication domains. This chromosome was arranged so the centromere was positioned towards the periphery and the telomeres towards the interior of the nucleus. Again the late replicating domains of chromosome 12 were also found towards the periphery of the nucleus, while early replicating bands were positioned more towards the interior (Nogami et al., 2000).

1.3.3 Relationship of chromosome arrangement to gene content and activity

The earliest evidence for chromosome position being dependent of gene activity was the inactive X chromosome, which forms a Barr body at the periphery of the cell nucleus. There is also good evidence that in human interphase nuclei, gene rich chromosomes lie towards the centre and gene poor chromosomes are at the periphery irrespective of chromosome size (Boyle et al., 2001). Chromosome paints were used to show that chromosome 19 had a more central location whereas chromosome 18 was positioned at the nuclear periphery at G_1 (Croft et al., 1999).

The more peripherally located chromosome 18, although it is larger than chromosome 19 and has a mean area 10% greater, has a smaller chromosome area in the interphase cell when compared to chromosome 19. The effect of changes in

transcription and histone acetylation on chromosome arrangement was also studied. AMD, an irreversible inhibitor of RNA polymerases caused a difference in the area of chromosome 19, but not chromosome 18. A histone deacetylase inhibitor TSA was also used to enhance the differences of expression between chromosome 18 and 19. However, while these treatments affected the compaction characteristics of the chromosomes, the chromosome positions were not altered.

Thus it is now clear that the position a chromosome depends on gene content and gene expression. Actively transcribed chromosomes, such as the active X chromosome and chromosome 19 are positioned in the interior of the cell nucleus and have more diffuse chromosome territories with a greater surface area, presumably allowing easier access for transcription by macromolecular complexes. Inactive chromosomes are much more compact and are positioned at the periphery of the nucleus (Eils et al., 1996).

1.3.4 Movement of chromosomes during the cell cycle.

One of the confusing factors in interpreting interphase chromosome positions was the prospect that chromosome position could depend on cell cycle stage. For example, centromeres are positioned non-randomly in interphase cells, but their position changes during the cell cycle. At G_1 centromeres are peripheral, with the chromosome arms orientated towards the interior of the nucleus, but at G_2 centromeres are positioned at the interior with the arms orientated towards the periphery (Ferguson and Ward, 1992, Popp et al., 1990). Early studies avoided variation introduced by cycle phase by studying neurons and other cell types, which were not actively dividing (Manuelidis and Borden, 1988, Nagele et al., 1999).

Position of chromosomes during the cell cycle was studied in synchronized cells by chromosome painting. In actively dividing human cells, chromosome 18 moved from the periphery of the cell nucleus to the interior of the cell nucleus during S phase and was repositioned at the periphery of the nucleus during G_1 after S phase. It was observed that there is a 2-4 hour period at the beginning of G_1 when the chromosome order was established (Bridger et al., 2000). Thus cell cycle does affect chromosome position and must be taken into account when studying interphase nuclear organization.

1.4 Nuclear organization in gametes and the zygote

It is unknown how the chromosome arrangement is established in the zygote. The arrangement may be set up from scratch within the zygote or a specific chromosome arrangement could be passed down from the parent through the germ cells to gametes and to the zygote.

Meiosis is the process by which haploid gametes are formed, and fertilization the process by which the haploid egg and sperm combine to produce the zygote. Nuclear organization passed on through meiosis may be passed on to the zygote. The retention/inheritance of a chromosome arrangement may have important implications for the expression of genes within the zygote. Therefore my study has concentrated on chromosome organization in sperm and male meiosis. Through this section I will discuss the nuclear organization during meiosis, spermatogenesis and in the zygote.

1.4.1 Meiosis

Meiosis represents a modification of mitosis, in which there is one round of DNA synthesis and two cell divisions. Whereas mitosis produces cells that are genetically identical to the parental cell, in meiosis random assortment of parental homologues, and recombination occurs between the parental chromosomes varies the genetic make-up of the haploid genomes being packaged into sperm.

Meiosis can be broken into four main cell types, spermatogonia, spermatocytes, spermatids and spermatozoa (figure 1.3).

At the beginning of the meiotic process, diploid spermatogonia undergo successive mitotic divisions before differentiating into primary spermatocytes. Spermatogonia have two main purposes, one, to produce more spermatogonia allowing the continuation of the meiotic process and two, to differentiate into spermatocytes. (reviewed by Weiss, 1983).

The first stage of meiosis begins with the differentiation of spermatogonia into primary spermatocytes. These cells enter an extended prophase 1 stage that consists of stages preleptotene – leptotene – zygotene – pachytene – diplotene. The primary spermatocytes firstly undergo replication and the chromosomes then begin to condense, and this progresses over all prophase 1 stages. Homologous chromosomes pair and recombine at leptotene, and by pachytene homologous chromosomes have paired side by side to form a bivalent composed of four chromatids. Synapsis is brought about via a

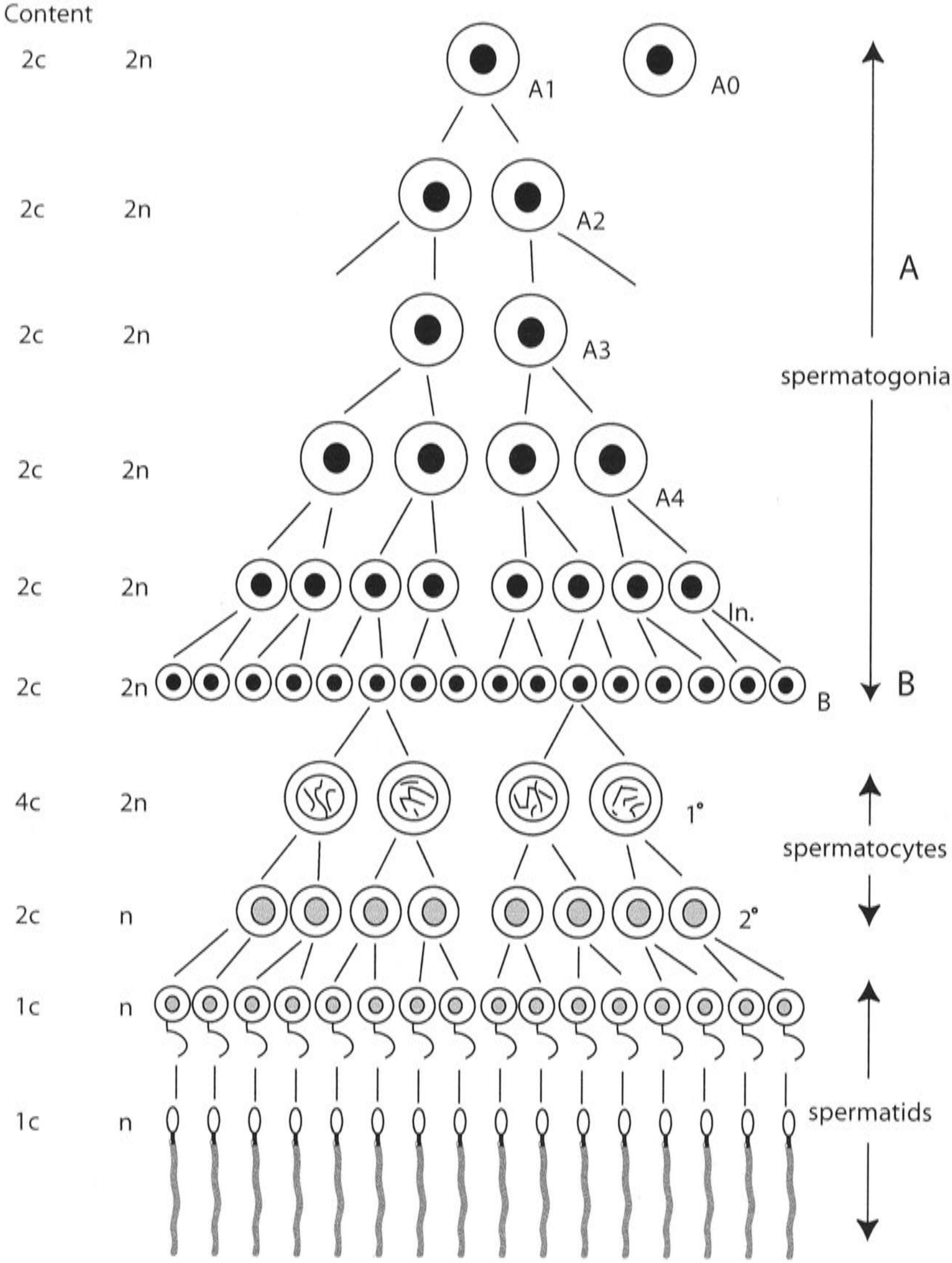


Figure 1.3: Mammalian spermatogenesis. Spermatogenesis in rat testis. Each cell level is interconnected with intercellular bridges that allow communication and exchange of gene products and nutrients. Spermatogonia A divide by mitosis to produce more spermatogonia A, and then Spermatogonia B, which enter meiosis, differentiate into primary spermatocytes, and divide producing four haploid spermatids that subsequently mature into sperm.

protein structure (the synaptonemal complex) that binds the two homologues. Crossing over occurs between chromosomes where genetic information is exchanged varying the genomic distribution of the haploid genome. The ladder like synaptonemal complex (SC) is made up of a central element and two lateral elements.

Pachytene cells show one of the first examples of meiotic chromosome organization with the X and Y chromosome forming a sex vesicle (SV) at the periphery of the nucleus. The SV does not undergo recombination or pairing, and only forms a SC at the pseudoautosomal regions (PAR) in mouse and human.

Over the next stage, pachytene chromosomes shorten, thicken and continue into the next stage, diplotene, where condensation and cross-overs (chiasmata) can be observed migrating towards the end of the bivalent (terminalization). During this stage the synaptonemal complex breaks down, and homologous chromosomes are held together by chiasmata. Centromeres of homologous chromosomes are then pulled away from each other in the next stage, diakenesis. From this stage paired chromosomes (bivalents) take up their positions on the metaphase plate for segregation (reviewed by Gilbert, 1997).

In anaphase 1 the homologous chromosomes (each double) are separated to different poles and at telophase 1 two daughter cells are formed (haploid secondary spermatocytes with one set of chromosomes each duplicated). The two daughter cells pause briefly before the second division takes place. During the second division (essentially a mitosis) sister chromatids are separated at anaphase 2 forming spermatids.

1.4.2 Spermiogenesis

Whereas spermatogenesis refers to the whole process of sperm formation, from spermatgonia to spermatozoa, spermiogenesis refers to the differentiation of round spermatids into spermatozoa. Some of the changes that spermatids undergo include a change in morphology and exchange of histones for protamines. The haploid spermatids are joined by cytoplasmic bridges that enable gene products made in one cell to pass into other cells. As spermatids are haploid, half carry an X and half a Y. If genes on the X and Y chromosomes are expressed and needed for sperm development then the cytoplasmic bridges are essential to allow gene products to be passed between cells. Spermatids therefore act like diploid cells.

For fertilization to take place, sperm must be able to travel, meet and bind to the egg. The first step of mammalian sperm development is the construction of the acrosomal vesicle from the Golgi apparatus. This forms a cap over the mature sperm, and is used to bind to the egg. On the other side of the nucleus the centriole starts to form the flagellum. The morphology of the sperm nucleus may change. For example, the nucleus forms an arrow shape in some marsupials, and a fibrilliar shape in monotremes.

1.4.2.1 Sperm structure

All sperm regardless of the morphology consist of three different sections; the tail, midpiece and spermhead (figure 1.4). Each section fulfils a specific function essential for the fertility of the sperm.

The tail is used to propel the sperm to the egg. The tail includes the axoneme at the centre surrounded by 9 dense actin fibres that run longitudinally down the tail. This is surrounded by a sheath of continuous circumferential fibres with opposed longitudinal columns and a plasmalemma (figure 1.4a).

The midpiece includes the connecting region between the tail and spermhead, remnants of the centrioles, and the mitochondria that provide the energy essential for sperm motility. At the centre of the midpiece are the axoneme and the nine dense fibres that are surrounded by the mitochondria (figure 1.4b).

The spermhead contains the haploid nucleus surrounded by a membrane. The acrosome, which is situated on the spermhead, allows recognition, binding and penetration of the sperm into the egg. The acrosome consists of an outer and inner membrane and contains glycoproteins and numerous lysosomal enzymes essential for fertilization (figure 1.4c). On contact with the egg, the acrosome breaks down, releasing these enzymes to break down the localized region of the zona pellucida.

1.4.2.2 The nucleohistone to nucleoprotamine process

Protamines are simple positively charged proteins that bind tightly to the negatively charged DNA (Oliva and Dixon, 1991). In eutherian sperm, there are disulfide bonds between cysteine residues of different protamines, which leads to extremely condensed DNA and extremely stable chromatin. Protamine packaging of sperm DNA allows it to be packaged tightly into the small sperm nucleus, and protects the DNA from damage the sperm may incur during motility. This tight packaging makes it very hard for chromosome paints and probes to penetrate the sperm nucleus and hybridize to the DNA. Numerous chemical procedures must be used to decondense

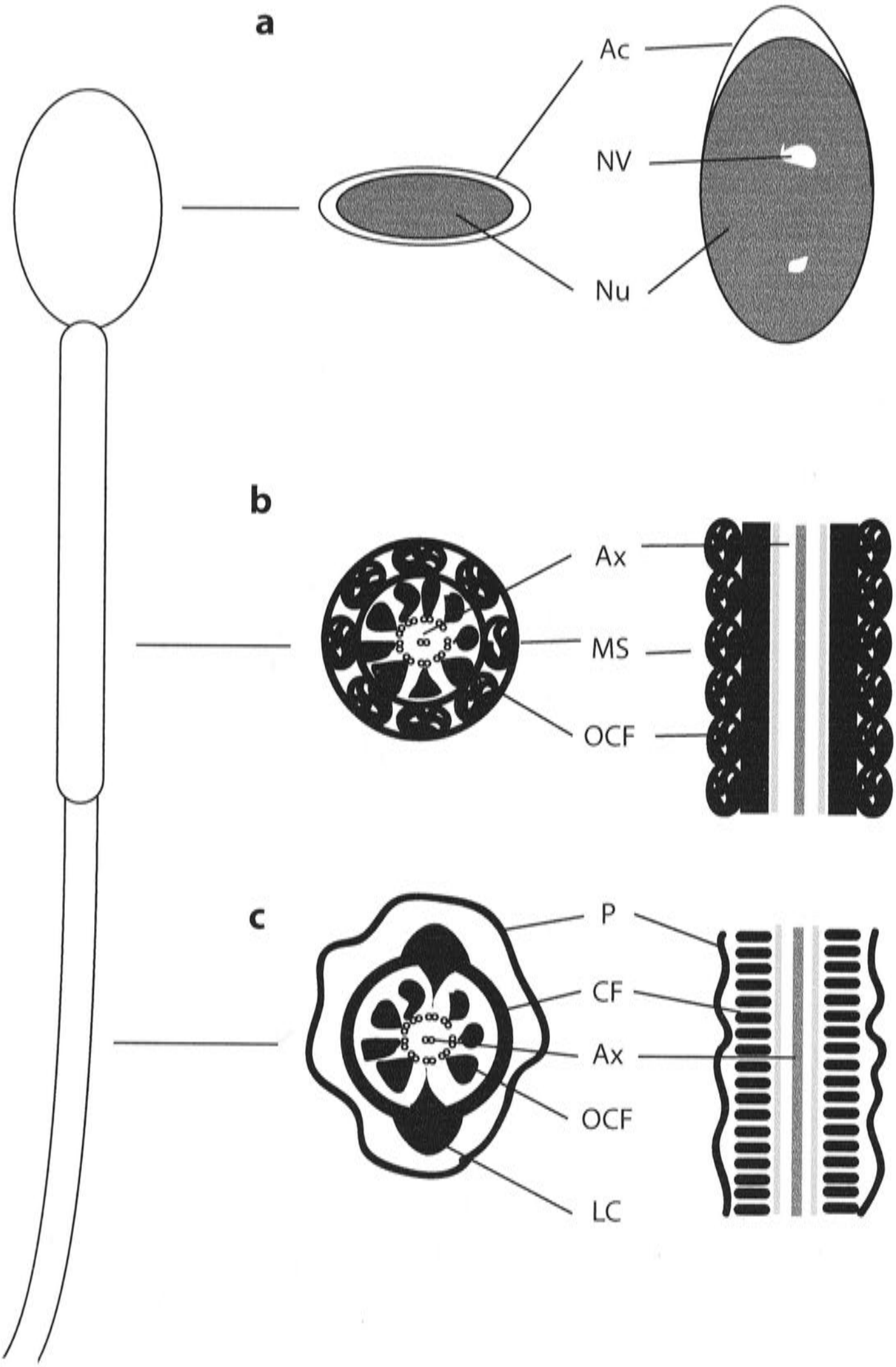


Figure 1.4: Basic sperm structure. Structure of the sperm head (a), midpiece (b) and tail (c) **Ac** Acrosome, **Nu** Nucleus, **NV** Nuclear vacoule, **LC** Longitudinal columns, **MS** Mitochondial shealth, **Ax** Axenome, **OCF** Outer course fibres, **CF** Circumferential fibres , **P** Plasmalemma.

the sperm nucleus for hybridization to occur, and this introduces the risk of artefact.

The displacement of histones and replacement by protamines begins with histone acetylation. This reduces the positive charge of the histones weakening the nucleosome-DNA association, unwinding and opening up the DNA, allowing protamines to bind (reviewed by Turner, 2000). There are two phases in the binding of protamines to DNA. In the first phase, phosphorylated protamines move along the DNA until the correct protamine binding position is recognized. Once the protamine has taken its position on the DNA, it is dephosphorylated and the DNA binds around it. This process occurs in a highly controlled and ordered manner, allowing proper condensation of the sperm nucleus (Oliva and Dixon, 1991).

Ubiquitination levels increase on histones H2A and H3 during spermatogenesis just before protamines displace histones. Ubiquitin is a protein found in all eukaryotes, that forms a variety of complexes with other proteins. One function of ubiquitin is to act as a targeting protein for the degradation of proteins via an ATP-dependent process. In spermatozoa it may help break the nucleosome-DNA association by targeting the histones for ATP-dependent proteolysis and allowing protamines to interact with the DNA (Oliva and Dixon, 1991).

1.4.3 Fertilization

Fertilization of the egg by the sperm is a complex process. Steps involve motility, activation of the sperm within the female duct (capacitation), recognition and binding of sperm and egg proteins, and fusion of the two pronuclei. In mammals, sperm are transported to the oviduct through swimming and the muscular activity of the uterus. Capacitation of the sperm then occurs. This is a necessary process that involves altering the sperm membrane by changing of the lipid composition by removing cholesterol, carbohydrates and proteins, which are thought to mask recognition of the binding site on the egg (Abou-Haila and Tulsiani, 2000). Proteins that are required for binding the sperm to the zona pellucida are activated by phosphorylation in a cAMP-dependant pathway. Capacitation results in a lowered membrane potential of the sperm (Abou-Haila and Tulsiani, 2000).

The egg is surrounded by the zona pellucida, which is essential for species-specific sperm recognition, binding and initiating the acrosome reaction. The zona pellucida contains a protein (ZP3) that binds the spermhead to the egg, initiating the

acrosome reaction. ZP3 is thought to bind to at least three proteins on mouse sperm, one of which specifically binds to the galactose residues of ZP3. The second protein is a sperm membrane glycosyltransferase enzyme, which causes the activation of a G protein that may be important in initiating the acrosome reaction. The third protein is a zona receptor kinase, which may initiate the acrosome reaction by phosphorylating target proteins (Primakoff and Myles, 2002).

Binding of the sperm to the zona pellucida causes the calcium ion channels on the sperm to open increasing the concentration of calcium within the sperm. This results in initiation of the acrosome reaction. Secondary binding between the inner acrosomal membrane of capacitated sperm and the ZP2 protein of the egg then occurs.

Once binding to ZP2 has taken place, fusion occurs between the sperm and egg membranes. The entry of the sperm causes actin polymerization, and elongation of several surface microvillii to form a fertilization cone, where the mitochondria, nucleus, centriole and flagellum will enter the egg once the membranes have fused. In sea urchin fertilization, the electrical potential of the egg membrane changes rapidly, 1-3 seconds after one sperm has fused and entered, by alteration of the concentration of potassium and sodium ions within the egg. A protease is expressed that specifically changes ZP2, preventing any other acrosome reacted sperm from moving further into the egg. Another protein produces an osmotic gradient, causing water to rush into the space between the cell membrane and the envelope, forming the fertilization membrane. Mammals lack a fertilization membrane, but they do modify the ZP2 to stop other sperm entering the egg. The mechanisms of preventing polyspermy differ in different species (reviewed by Weiss, 1983).

1.4.4 Arrangement of chromosomes in gametes and the zygote

Little is known about the arrangement of chromosomes in gametes and early developmental stages of the zygote. This is due to the difficulties in obtaining the right cell stages, and in the case of the zygote, obtaining enough samples to make an accurate interpretation of the chromosome arrangement. Little if any work is present on chromosome arrangement during meiosis, but there is literature on chromosome arrangements in sperm (chapters 3 and 5). A lot of work on chromosome organization in sperm was done in the 1970's, where methods such as C-banding and Feulgen staining provided inconsistent results. However, the recent use of chromosome paints has now suggested that a non-random chromosome arrangement is a characteristic of

mammalian sperm. Little work has been done on chromosome arrangement in egg, again due to limitations of obtaining a large enough sample size, also the amount of cytoplasm within the egg limits the ability of visualizing signals and any techniques used to remove the cytoplasm would affect the three dimensional structure of the egg destroying any nuclear organization present.

Little is known about chromosome organization before the fusion (syngamy) of the maternal and paternal pronuclei, but there is differential acetylation of paternal/maternal pronuclei in mice (Adenot et al., 1997). Upon decondensation of the sperm there is hyperacetylation of the male pronucleus. During this time the maternal pronucleus is at metaphase II and is hypoacetylated. The differential acetylation of the two pronuclei may set up and allow differential imprinting of the paternal pronucleus, setting up paternal X inactivation.

Chromosome arrangement has been studied in mouse zygotes using α-satellite, telomeric and whole chromosome paints after syngamy has occurred (Dozortsev et al., 2000). It was found that although the X chromosome and chromosome 1 were juxtaposed, chromosome positions appeared to be random. However, the authors suggested that this might be due to the collapse of the three-dimensional structure. Centromeres came together with the nucleolar organizing region (NOR) to form a shape reminiscent of spokes on a wheel. This structure is similar to that seen in human and mouse sperm, in which centromeres come together to form a chromocentre, with the telomeres at the periphery (Zalensky et al., 1995). The configuration is present in sperm when it enters the egg; it goes through decondensation and re-forms within the pronucleus. This suggests that chromosome arrangement in sperm is retained at least until pronuclei come together in syngamy. The ordered arrangement may therefore be critical in setting up a normal arrangement of chromosomes in the embryo.

Other studies compared the positions of early and late replication sites rather than chromosomes in mouse zygotes. As in somatic mammalian cells, early and late replicating sequences are separated in the pronuclei of the zygote (reviewed by Spector, 1993), and specific regions of DNA occupy defined regions in the nucleus that replicate at precise times during S-phase (Ferreira et al., 1997). Thus there is similar organization in adult cells and zygotes in the spatial and temporal replication of DNA. Replication timing between the maternal and paternal pronuclei was asynchronous.

DNA methylation is a common imprinting mechanism in identifying and inactivating either the maternal or paternal genome. In humans, chromosomes 11p15.5

and 15q are imprinting regions where either the maternal or paternal copy of the gene is switched off. A lot of the genes involved in imprinting are developmentally important such as *Igf2* and *H19*. At 3 hours after fertilization the maternal and paternal chromosomes are equally methylated. At 8 hours after fertilization there is undermethylation of the paternal genome compared to the maternal genome. This differential methylation between the maternal and paternal genome can be seen beyond the four cell stage (reviewed by Haaf, 2001). The fact that such differential patterning of the maternal/paternal genomes is present highlights how the paternal X chromosome could be distinguished for inactivation from the maternal chromosome.

1.5 Nuclear organization and gene activity

1.5.1 Gene organization in the interphase nucleus

It is therefore evident that chromosomes have specific positions that are related to their gene content and activity (figure 1.5a), and that organization of the chromosome territory is separated into early and late replicating domains (figure 1.5b). Early replicating DNA contains active genes, whereas late replicating DNA is associated with heterochromatin and inactive genes. Thus active genes are associated together and kept separate from the inactive and heterochromatic DNA.

Differentiation involves the activation of a certain subset of genes and the inactivation of other genes. The active genes are maintained in a decondensed state, whereas inactive genes are maintained in a condensed state (reviewed by Francastel et al., 2000). The amount of condensed chromatin differs between different cell types, showing that nuclear organization and expression of certain genes is cell type dependent (Leitch, 2000).

In hemopoietic cells, genes relevant for the many different future lineages are transcribed and kept in open chromatin configurations before the cell fate has been determined (Hu et al., 1997). After the cell fate has been determined, all genes except those transcribed in the cell lineage are permanently turned off. This deactivation of genes involves chromatin condensation, deacetylation of histone 3 and 4 and accumulation of linker histones (Crane-Robinson, 1999).

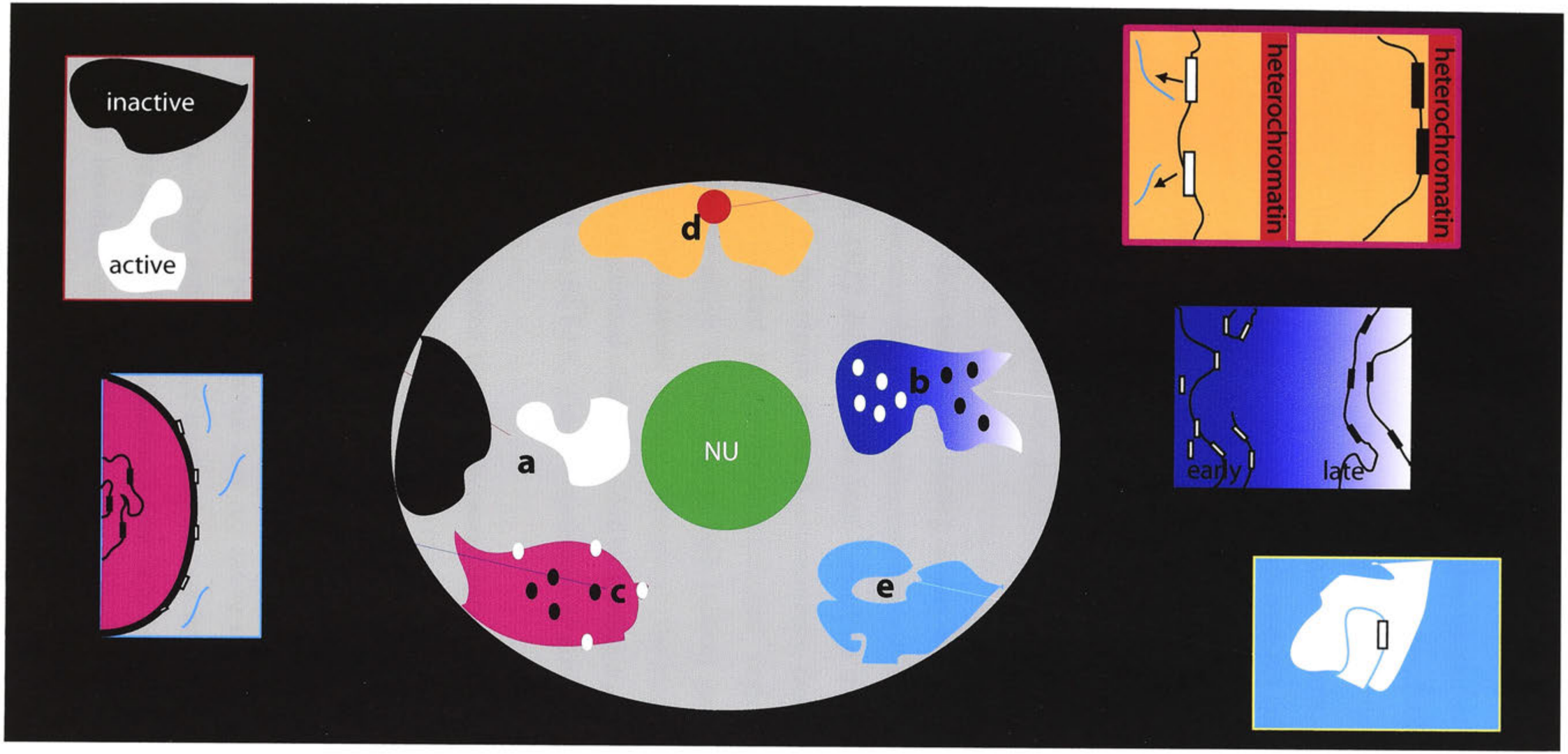


Figure 1.5: Interphase nuclear organization where chromosomes form distinct territories. a) Chromosomes with a high gene content are found in the interior of the nucleus whereas gene poor chromosomes, or inactive chromosomes are peripheral. b) Chromosomes have different regions of late and early replicating DNA. Early replicating genes lie towards the interior of the nucleus and late replicating genes are towards the periphery. c) Active genes lie towards the periphery of the chromosome territory and inactive genes are in the interior. d) Genes are 'switched off' when situated close to heterochromatin. e) Active genes may be moved into interchromatin domains where they can be transcribed. ▭ = active genes ▬ = inactive genes

It is known that active genes are found at the periphery of chromosome territories, whereas inactive genes are located at the interior of chromosome territories close to heterochromatin (reviewed by Cremer and Cremer, 2001) (figure 1.5c). Heterochromatin has long been thought to promote gene silencing, and FISH localization in interphase cells showed that inactive genes were associated with centromeric heterochromatin, whereas active genes were not (Brown et al., 1997). This was also seen for the human *SOX-1* and *C - FMS* genes, which were associated with pericentromeric heterochromatin when inactive, but not when active (Brown et al., 2001).

The three immunoglobulin genes, κ, λ and γ, are differentially and non-randomly distributed in different sub-nuclear regions (Parreira et al., 1997), but their position does not change in different cell types in which one or the other is expressed. One explanation for this unexpected finding is that the amount of heterochromatin surrounding the gene may change depending on activity of a gene (figure 1.5d).

The genes *ANT2* and *ANT3* are both located on the human X chromosome. *ANT2* is situated in the region of the X chromosome that undergoes inactivation, whereas *ANT3* is located in the pseudoautosomal region (PAR) that escapes inactivation. On the active X chromosome both (active) genes are positioned at the periphery of the X chromosome territory, whereas on the inactive X chromosome *ANT 2* (located in the X inactivated area) is found in the central region of the X chromosome) and *ANT3* (located on the PAR, which remains active) remains at the periphery of the cell territory.

It has been suggested that gaps between chromosome territories may occur in which genes can be transcribed (reviewed by Cremer and Cremer, 2001). These gaps, called interchromatin domains, allow macromolecular complexes to move into regions of the cell nucleus that allow transcription to occur. These domains may be dynamic, appearing or disappearing when genes are activated or inactivated. Therefore genes need not change their position when they are active if interchromatin domains form (figure 1.5e). Thus activation or inactivation may be accomplished by moving the interchromatin domains or heterochromatin relative to genes.

It is becoming quite evident that chromosome position is dependent on gene activity, or that gene activity is dependent on chromosome position. Furthermore the organization of the chromosome territory itself seems to be dependent on gene activity.

1.5.2 Conservation of chromosome organization

The conservation of chromosome organization might be a good indication of the importance of chromosome position in cell function. If the arrangement of homologous chromosomes is conserved in different species this would point to chromosome position being important in cell function. Chromosome arrangement has recently been compared in primates (Tanabe et al., 2002), using the homologues of human chromosomes 18 and 19, which are comparable in size, but have different gene densities. The positions of homologous chromosomes were studied in S phase Epstein-Barr virus-transformed lymphoblastoid cell lines from chimpanzees, gorillas, orangutans, white-handed gibbons, cotton-top tamarins, common marmosets and squirrel monkeys (Tanabe et al., 2002). These primates include species with karyotypes very similar to humans (chimps, gorillas), species with some changes (new world monkeys) and species with a high degree of chromosome reshuffling (Gibbons) (Jauch et al., 1992, Stanyon et al., 2001, Yunis and Prakash, 1982).

When human chromosome 18 and 19 paints were hybridized to chromosome preparations of the other primates' preparations, they hybridized to homologous chromosomes. At interphase the signals observed were in the same relative position in all species, although both chromosomes were positioned more towards the periphery of the nucleus in chimpanzees, consistent with the observation that chimpanzee chromosome 18 and 19 have more heterochromatin than the human equivalents. Conservation of chromosome position between other species will help us assess the importance of chromosome organization.

1.5.3 Chromosome organization and disease

If chromosome arrangement is important in the regulation of gene activity, disruptions of arrangement could cause abnormal gene activity and be expressed as disease. Very little is known about the role of chromosome positioning in disease. As yet there is no example of an abnormal chromosome arrangement being the direct cause of disease, although there are examples of abnormal chromosome arrangements in diseases.

The best documented case of abnormal chromosome positioning is that of epilepsy. Chromosome-specific paints were used in conjunction with serial sectioning and electron microscopy to compare positions of chromosomes 1, 9, the Y and X

chromosomes in neurons from normal males and females to neurons in electrophysiologically defined seizure foci of epilepsy patients (Borden and Manuelidis, 1988). It was determined that the X chromosome had taken a more internal position in seizure foci than normal cells in both males and females. Slight differences were also observed in positions of chromosome 9, but not in the Y chromosome. It is possible, therefore, that abnormal positioning or abnormal chromatin packaging of the X chromosome may change gene expressions and cellular functions, resulting in seizures (Borden and Manuelidis, 1988). The more interior positioning of the X chromosome in seizure foci suggests higher levels of expression of genes on the X chromosome, since gene rich and active chromosomes are positioned more centrally. A higher level of gene expression on the X chromosome in seizure foci may contribute to the syndrome or even cause it. It may be significant that the human X chromosome has a two-fold enrichment of genes involved in brain development and function (Zechner et al., 2001), and over-expression of one or more of these genes may produce seizures.

There is also evidence that relationships between homologous chromosomes may be altered in some diseases. There is normally an association between the imprinted regions of the two chromosome 15 homologues at 15q11-q13 (LaSalle and Lalande, 1996) (an exception to the lack of homologous association discussed in 1.2.1). However, in S phase cells from patients with Prader-Willi and Angelman syndromes, there is a lack of association between the imprinted regions. This suggests that the imprinted region of chromosomes is important for recognition and association.

1.5.4 X-inactivation

The inactive X chromosome (Xi) follows the rule that gene rich chromosomes and active genes lie towards the interior of the nucleus, and gene poor chromosomes and inactive genes lie towards the periphery of the nucleus. Indeed, the inactive X chromosome was the prototype of this rule, with the early observation of a sex chromatin body in neurons of female, but not male cats (Barr and Bertram, 1949). This condensed body was also observed at the periphery of the cell nucleus in human female fibroblasts. X-inactivation still provides the best known correlation between nuclear organization and gene activity.

Inactivation of the X chromosome results in dosage compensation, allowing equal expression rates of X-borne genes in XY males XX females. This is necessary because the mammal X chromosome contains 1400 genes that are not found on the

small, heterochromatic Y chromosome. Although the X and Y chromosomes evolved from an autosomal pair, the Y chromosome seems to have degraded, losing all of the genes except about 50, many of which are directly or indirectly involved in sex determination (reviewed by Graves and Shetty, 2000).

1.5.4.1 Initiation of X-inactivation

Eutherian X-inactivation involves the initiation and then maintenance of the inactive state, whose extraordinary stability relies on a complex of different mechanisms. Inactivation is due to transcriptional repression (Graves and Gartler, 1986). This is achieved by a multi-step mechanism (Gartler et al., 1985) involving hypermethylation of the inactive X chromosome at CpG islands (Pfeifer and Tonguay, 1990), enrichment in macro Histone 2A (Costanzi and Pehrson, 1998), reduced acetylation of histone 3 (Boggs et al., 1996) and hypoacetylation of histone 4 (Jeppesen and Turner, 1993).

Initiation of X-inactivation begins at the X inactivation centre (XIC), which includes the X inactive specific transcript (*XIST*) gene and its antisense transcript *TSIX*. *XIST* transcribes a non-coding RNA that coats one X chromosome, presumably keeping it inactive.

Eutherian X-inactivation begins by "counting" the numbers of X chromosomes and then "choice" of the X chromosome to be inactivated. The cell is able to count the number of X chromosomes (n) and inactivate all but one (n-1). If there is one X chromosome then there is no X-inactivation, if there are two X chromosomes then one is inactivated, if there are three X chromosomes then two will be inactivated.

Choice of which X chromosome will be inactivated depends on the expression of *Tsix*, the antisense transcript of *Xist*. *Tsix* is initiated downstream from *Xist* and prevents the inactivation of one of the X chromosomes (Lee et al., 1999). Expression of *Tsix* may depend on a 3kb CpG region found in its initiation site, which is hypermethylated on the active X chromosome (reviewed by Mlynarczyk and Panning, 2000).

1.5.4.2 maintenance of X-inactivation

Once the X chromosome has become inactive, many different mechanisms help provide a stable inactive state. During interphase, the inactive (but not the active) X chromosome has a characteristic loop structure, with the telomeres in close proximity (Walker et al., 1991). During mitosis the inactive X chromosome has a characteristic kink. Localization of macroH2A (mH2A) to the inactive X chromosome also plays

important roles in producing the inactive heterochromatic state (Costanzi and Pehrson, 1998). Histone mH2A will be further discussed in section 1.6.

Observations on the inactive X chromosome demonstrated that it was late replicating (Willard and Latt, 1976). Thus the Xi follows the rule that inactive genes replicate late (discussed in section 1.4.1), even within the Xi this correlation holds. Regions of the inactive X chromosome, which escape inactivation, include the pseudoautosomal region and several genes with Y homologues, all of which replicate earlier (Schempp and Meer, 1983). It is not known if this late replication is a cause or an effect, or a correlation of X-inactivation and maintenance, but it seems likely that it is a byproduct of the heterochromatic and inactive state of the DNA.

One of the most important mechanisms conferring stability of inactivation is the methylation of CpG islands in DNA on the inactive X chromosome, as shown by the effect of demethylation of DNA in reactivating the X chromosome (Graves, 1982, Mohandas et al., 1981). Methylation of CpG islands upstream from genes reduces transcription levels by preventing binding of transcription factors. Bisulphite sequencing (that reveals positions of methylation) showed that CpG islands in the PGK-1 promoter region were highly methylated on the inactive X chromosome, whereas methylation was not present in the corresponding region of the active X chromosome (Pfeifer and Tonguay, 1990).

Using DNA footprinting to study the promoter region of the human PGK-1 gene, it was possible to determine the DNA sequence in the PGK promoter to which a particular DNA-binding protein binds. Footprinting the active X chromosome showed eight regions of protein-DNA contacts. Regions on the inactive X containing consensus sequences for transcription factors such as ATF and NF1 were methylated, indicating that methylation inhibits the binding of transcription factors to the promoters, and the initiation of transcription.

Although X-linked genes are methylated on the inactive X chromosome, methylation itself is not required for inactivation in all mammals. However, methylation of CpG islands 5' of *Hprt* on the inactive X chromosome in rodent embryos is not methylated until several days after X-inactivation has occurred (Lock et al., 1987). This indicates that methylation plays an important role in the stability and maintenance of eutherian X-inactivation but not in the initiation of X-inactivation.

A much earlier change in the human and mouse inactive X is histone deacetylation. When human female metaphase spread preparations were immunostained

with H4ac and H3ac antibodies, the whole inactive X chromosome was hypoacetylated except for acetylation bands observed at the two PAR regions (Jeppesen and Turner, 1993).

Thus eutherian X-inactivation involves a complex of inactivating mechanisms, including *XIST* expression, heterochromatinization, late replication, mH2A localization and deacetylation of the inactive X chromosome, to initiate and then maintain X-inactivation. Marsupial X chromosome inactivation is phenotypically distant, and seems to require a simple mechanism. In this study I compare some of the molecular changes in the marsupial X to deduce which mechanisms are conserved and therefore ancestral in mammalian X-inactivation.

1.6 Chromatin organization and activity

The highly organized gene and chromosome arrangement suggests that it serves an important function. Function may be more apparent at the level of chromatin structure, since changes in chromosome structure and gene expression reflect changes in binding of DNA to chromosomal protein. Reversible changes in gene expression occur without alterations to the DNA sequence. One mechanism for this differential expression of genes is the positioning of a gene in a heterochromatic region of the cell nucleus.

Mammalian cells contain a huge amount of DNA. The human haploid genome size is 3.3×10^9 base pairs, so that each human cell contains around 2 metres of DNA contained within a nucleus with an average nuclear diameter of 6μm. Thus cells must package the DNA efficiently to enable it to fit into a cell. This is done through the binding of DNA with proteins that coil and supercoil the DNA.

The most important nuclear proteins belong to a family of basic proteins called histones. Histones have a high proportion of positively charged amino acids (arginine and lysine), which tightly bind to the negatively charged DNA (Alberts et al., 1983). Histones are among the most highly conserved proteins known with just one amino acid sequence difference between peas and cows (Griffiths et al., 1996). The major types of histones are histone 1 (H1), histone 2A (H2A), histone 2B (H2B), histone 3 (H3) and histone 4 (H4) that are arranged in ordered structures called nucleosomes. Changes in gene expression may require changes to interactions between these proteins and DNA.

1.6.1 Chromatin structure: Nucleosomes and solenoids

DNA is wrapped around nucleosomes to form 'Beads on a string' when observed with an electron microscope (Kornberg, 1974). The 2nm DNA strand is wrapped twice around a single nucleosome made up of eight histone proteins. Two H4, H3, H2A and H2B make up the nucleosome. The nucleosome structure involves a $(H3/H4)_2$ tetramer, on either side of which is bound a H2A/H2B dimer (Luger et al., 1997). This creates a left-handed superhelical ramp of protein with almost 160 base pairs of DNA wrapped around it (Arents et al., 1991). Each nucleosome is separated by 60 base pairs of "linker" DNA bound by histone H1. The linker DNA and the nucleosome together constitute 220 base pairs. Contact between the nucleosomal histones and the DNA involves arginine residues penetrating the minor groove of DNA and the interactions of several polypeptide chains which interact with two consecutive phosphates on each DNA strand (Arents and Moudrianakis, 1993).

The next level of structure involves six nucleosomes that come together to form a 30nm solenoid. Histone 1 (the "linker" histone) brings nucleosomes together to form the 30nm structure by binding to a specific region of the nucleosome, then extending to cover part of the linker DNA and join it to adjacent nucleosomes. H1 also binds to other H1 molecules forming clusters of six, which pulls the nucleosomes together to form the 30nm solenoids. Removal of H1 allows the solenoid to unwind.

1.6.2 Histone tails

At the N and C terminals of the histones are 'tail domains', which comprise 25% of the histone mass. They are less tightly bound to DNA and more accessible. The tails do not play an important role in nucleosome stability or positioning, but are important for nucleosome – nucleosome interactions, and are essential for making the 30nm fibre (Tse and Hansen, 1997). Histone tails are susceptible to modifications that affect chromatin structure, condensation and transcription.

Types of histone modifications to the N terminal tail of histones include acetylation, phosphorylation, ubiquitination, ADP-ribosylation and methylation. Each of these different modifications can drastically change the interactions between nucleosomes and DNA, affecting chromatin structure and transcription (reviewed by Wolffe and Hayes, 1999).

1.6.3 Histone modifications

1.6.3.1 Acetylation

Post-translational acetylation of histones has been intensively studied since it was first correlated with gene activity (Allfrey et al., 1964). Different histone 4 acetylation patterns are observed in euchromatin and heterochromatin (Turner et al., 1992). Acetylation reduces the positive charge of histones 3 and 4, weakening their association with DNA, and allowing macromolecular complexes to bind to the DNA and initiate transcription. The weakened association with DNA does not affect the winding of the DNA around the nucleosome, but does decrease nucleosome-nucleosome interactions (reviewed by Wolffe and Hayes, 1999).

The course of acetylation at different sites in histone 4 follows a set order of lysine 16, 8, 12 and lastly 5. Monoacetylated DNA is acetylated at lysine 8 or lysine 16, but never at lysine 5 or lysine 12 (Turner et al., 1989). Nucleosomal histones need to be acetylated at only 15 of a possible 28 lysine sites for transcription to be increased by over 15 fold (Tse et al., 1998).

Acetylation and deacetylation are carried out within transcriptional coactivator complexes and corepressor complexes that contain acetyltransferases and deacetylases (Brownell and Allis, 1996). Acetylation states of nucleosomes must be actively maintained as acetylation states have a half-life of only minutes, compared to the more stable hypoacetylated nucleosomes (Zhang and Nelson, 1988).

Staining with antibodies against histones acetylated at particular sites has shown that euchromatin can be acetylated at some or all of lysine residues 16, 8, 12, and 5, whereas heterochromatin is usually hypoacetylated (O'Neill and Turner, 1995). One of the best examples of the correlation between hypoacetylation and heterochromatin is the differential staining between the active and inactive X chromosomes in cells of human females which show that the active X is acetylated whereas the inactive X chromosome is hypoacetylated (Belyaev et al., 1996, Jeppesen and Turner, 1993). One of the X chromosomes is also hypoacetylated in cells from female marsupials (Wakefield et al., 1997). Although the *D.melangaster* dosage compensation system is very different, the single X chromosome in males is hyperacetylated to achieve elevated transcription (Turner et al., 1992).

1.6.3.2 Phosphorylation

Phosphorylation of histone 3 is important for the initiation of chromosome condensation at mitosis and meiosis. Phosphorylation of serine 10 in H3 occurs in

pericentromeric chromatin at late G_2, rapidly spreads throughout the chromosome just before mitotic prophase (Van Hooser et al., 1998) and is lost during anaphase (Hendzel et al., 1997). During meiosis, phosphorylation occurs at metaphase 1 and 2 (reviewed by Hans and Dimitrov, 2001). However, induction of H3 phosphorylation with Okadaic acid caused only slight chromosome condensation, showing that phosphorylation alone is not sufficient to cause full chromosome compaction. Similarly, dephosphorylation of mitotic cells using a mild hypotonic, did not cause decondensation of chromosomes, meaning that maintaining condensation does not depend on phosphorylation.

1.6.3.3 Ubiquitination

Ubiquitin is a small conserved protein consisting of 76 amino acids. It is found in all living organisms and is associated with many different processes including cell cycle control, protein degradation and DNA repair (reviewed by Jason et al., 2002). Ubiquitin links to lysine 119 at the face of the histone octomer in 10-15% of all histone 2A molecules. Ubiquitin is also bound at Lysine 120 of histone 2B, and histone 3 is ubiquitinated in elongating rat spermatids (Chen et al., 1998).

There is increasing debate over the role of histone ubiquitination in actively transcribing DNA. In *Drosophila* the level of ubiquitination is correlated with transcriptional activity, as half of the histone H2A of an actively transcribed gene (*hsp70*) was ubiquitinated, whereas a non-transcribed alpha-satellite had only one ubiquitinated H2A per 25 nucleosomes (Levinger and Varshavsky, 1982). However, an earlier study linked the loss of ubiquitination with an increase in transcription in rat liver nuclei (Ballal et al., 1975).

Histone ubiquitin levels have been studied at different stages in the cell cycle. High levels were found at interphase and early prophase, after which the levels started to decline and were undetectable at metaphase. The levels of ubiquitin started to rise again at anaphase and continued to rise (Mueller et al., 1985). The role of H2A and H2B ubiquitinization is not known. One possibility is that ubiquitin acts as a tag recognized by target proteins, causing chromatin and nucleosome degradation. Histone ubiquitin could also act in concert with histone acetylation, in a process by which acetylation destabilizes the nucleosome core, and ubiquitin weakens the interaction of the H2A-H2B dimers with H3-H4 tetramers (reviewed by Jason et al., 2002).

1.6.3.4 ADP-ribosylation

The basic energy of the cell is obtained through the hydrolysis of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) + phosphate (P) (Alberts et al.,

1983). However, ADP is also known to affect other cell functions. ADP-ribosylation has been implicated in DNA synthesis, DNA excision repair, gene expression and cell differentiation (Leone et al., 1985). ADP-ribose can be transferred to different proteins within the cell, affecting cell function and expression. An ADP-ribosyl molecule is transferred from the nicotinamide adenine dinucleotide enzyme (NAD) to other proteins (Creighton, 1983), such as histones, by Poly (ADP-ribose) synthetase.

The role of ADP-ribosylation of histones is unknown. In different studies, ADP-ribosylation has been associated with gene transcription, or shown to have the same levels in inactive and active chromatin. In one study, the interaction with histones and ADP-ribosylation was compared between active chromatin and inactive chromatin. The level of ADP-ribosylation of all histones was much greater (2-10 fold) in inactive chromatin than active chromatin (Tikoo and Ali, 1997).

1.6.3.5 Methylation

Methylation at the N terminus of histone 3 (lysine 4 and 9) and also histone 4 (arginine 3) has recently been found to influence transcription. (Boggs et al., 2002, Peters et al., 2002, Wang et al., 2001). Methylation of H3 at lysine 9 occurs predominantly in heterochromatin, whereas methylation of lysine 4 occurred in euchromatin. In human female cells, the inactive and active X chromosomes stained differently with an antibody to H3 methylated lysine 9 and lysine 4 (Boggs et al., 2002). The inactive X chromosome was enriched for H3 methylated at lysine 9, but hypomethylated at lysine 4. Regions of the inactive X corresponding to the pseudoautosomal region that escapes inactivation were enriched for lysine 4 methylation. Immunoprecipitation with the H3 lysine 4 methylated antibody detected only the *XIST* gene, which is transcribed from the inactive chromosome (Boggs et al., 2002). Methylation of histones on the inactive X chromosome was continued through mitosis, suggesting a stably propagated epigenetic mark for the inactive X chromosome.

Methylation of lysine 9 at H3 is maintained by site specific methyltransferase, Suv39h HMTase (Rea et al., 2000), the disruption of which abolishes H3-lys9 methylation of pericentric heterochromatin. However, this treatment does not disrupt the methylation of H3-Lys9 on the inactive X, suggesting that a different methyltransferase is involved in X inactivation (Peters et al., 2002). Treating cells with Trichostatin A (TSA) an inhibitor or deacetylases, increases histone acetylation within the cell, and decreases methylation of H3-Lys9. The inverse relationship between histone acetylation and methylation suggests that histones are not acetylated and

methylated at lysine 9 at the same time. HP-1 is a protein that binds to heterochromatin and has a role in keeping DNA in a heterochromatic state (Eissenberg and Elgin, 2000). Decrease in methylation of H3 lysine 9 causes disruption to the binding of HP-1. The pattern of H3-lys9 methylation and HP-1 binding was disrupted when Rnase A was added. This suggests that an RNA transcript (similar to *XIST*), as well as HP-1 and methylation at lysine 9 on H3, are needed to maintain heterochromatin (Maison et al., 2002).

Methylation of the histone termini is not always involved in silencing, since methylation of lysine 4 and of arginine 3 is involved in activation of transcription. Methylation of H4 arginine 3 is carried out by a different enzyme, arginine *N*-methyltransferase 1 (PRMT1). The finding that methylation at H4-arg3 occurs on hypoacetylated but not hyperacetylated H4 suggests that acetylation impairs PMRT1 recognition of H4 substrates (Wang et al., 2001).

1.6.4 The histone code

Thus histones that package DNA within a cell undergo important post-translational modifications that affect compaction, transcription and maintenance of the chromatin structure.

1.6.4.1 Patterns of histone modification

Histone modifications may work in concert to produce a histone code that would greatly extend the information potential of the genetic code (Jenuwein and Allis, 2001, Strahl and Allis, 2000, Turner, 2000). Different basic histone codes occur in different types of DNA. For example, the specific "code" for heterochromatin histone modifications would contain methylation of H3-lys 9 (figure 1.6a), whereas the specific "code" for euchromatin histone modifications would contain H3 and H4 acetylation.

There is also the possibility of combinations of different modifications, or of certain modifications blocking other modifications in close proximity. For instance, there is some evidence for an inverse relationship between histone phosphorylation and methylation. Phosphorylation of H3 serine 10 inhibits methylation of lysine 9, but correlates with acetylation of lysine 9 and lysine 14, marking the tail for transcriptional activation. It has also been observed that lysine 14 must be deacetylated for methylation of lysine 9. (Nakayama et al., 2001).

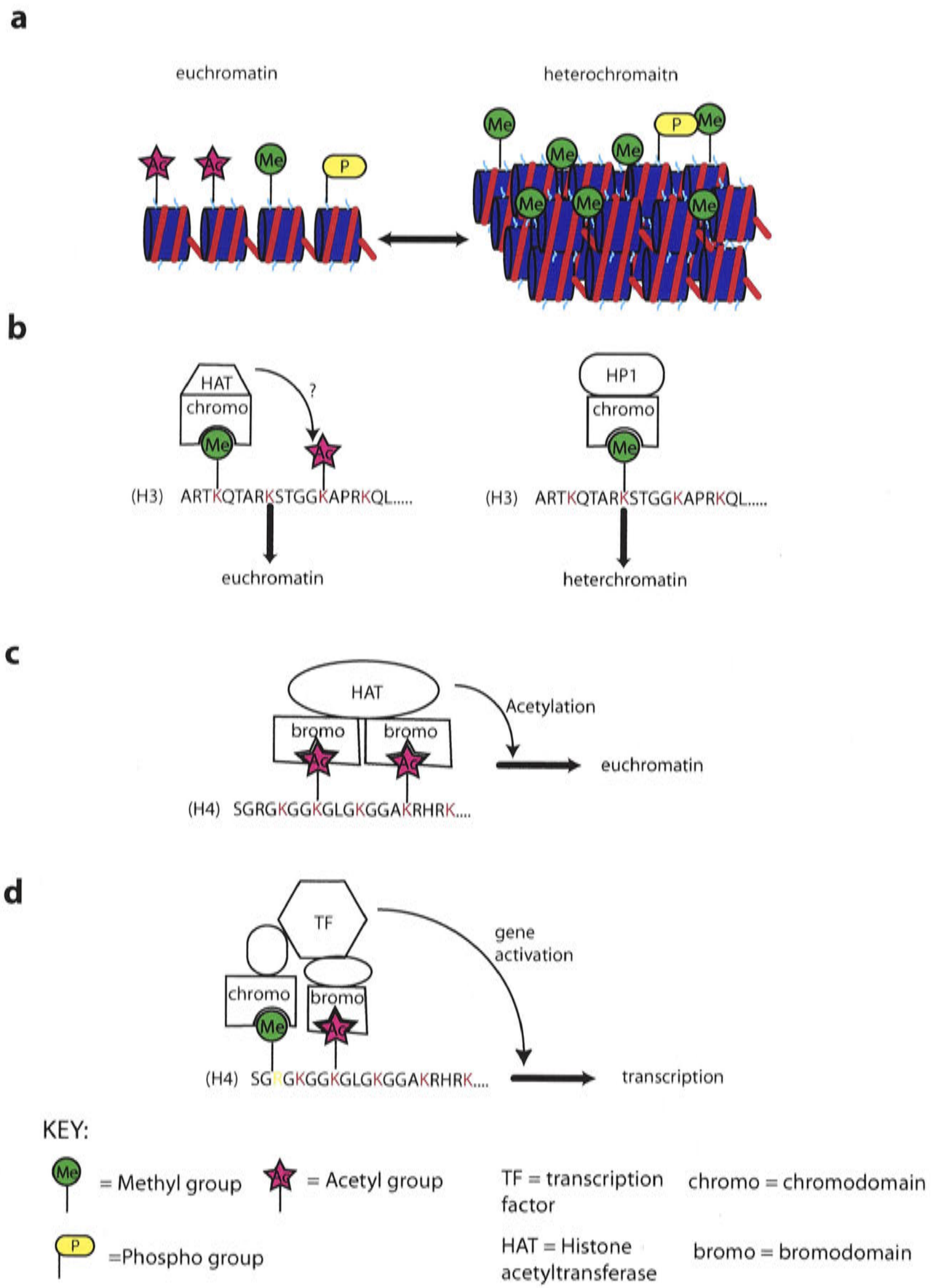


Figure 1.6: Relationship between histone modifications and chromosome condensation and activation. (a) Different modifications on nucleosomes in euchromatin and heterochromatin. (b) Differential methylation at different sites can inactivate or activate transcription. (c) Some proteins contain two or more bromodomains (methyl - binding regions of proteins) that help recognize modified nucleosomes associated with active DNA. (d) Different chromo and bromodomains may interact to affect transcription. (Modified from Jenuwein, 2001).

1.6.4.2 Inheritance of the histone code

Such a histone code would have to be established, maintained and passed through mitosis and meiosis, as has been observed for somatic inheritance of histone acetylation. Acetylation and phosphorylation of histones have a high turnover, whereas methylation is a comparatively stable modification. No histone demethylases have yet been discovered so methylation of histones may be critical in passing a transcription state on to the next generation of cells (reviewed by Jenuwein and Allis, 2001). It may be such a mechanism that allows the differential inactivation of the paternal and the maternal X chromosomes observed in marsupials. Therefore, the histone code may provide a mechanism for transmitting gene expression patterns between the parental and daughter cell (Jeppesen, 1997).

1.6.4.3 Proteins that maintain the histone code

The proteins involved in the maintenance of histone modifications include a group known as suppressors of variegation. Position effect variegation is the somatically heritable differences in the expression of a gene according to the accessibility of its chromatin environment. For instance, the wild type eye colour gene in *Drosophila* is active but is variably silenced when moved close to heterochromatin (reviewed by Schotta et al., 2003).

A number of genes suppress this variegation. These Su(var) prove to code for proteins involved in maintaining heterochromatin, including histone deacetylases (HDACS), protein phosphatases (PPTases), and heterochromatic protein 1 (HP-1 [Su(var)2-5]. There is also a group of modifiers E(var) that is antagonistic to the Su(var) proteins. This group commonly contains ATP-dependent remodeling proteins such as SWI/SNF and brahma complexes, all of which increase the mobility of the nucleosome (reviewed by Jenuwein and Allis, 2001). The Su(var) and E(var) proteins also contain bromodomains and chromodomains that are shared with other antagonizing chromatin regulators. These different domains help increase the functional diversity of genes involved in regulating gene expression and chromatin structure through histone modifications.

The Su(var)3-9 family contains histone methyltransferases (HMTases) with conserved SET domains. SET domains (130 amino acids) are small regions of larger proteins that read the tails of histones and place methyl groups at specific lysines (Yeates, 2002). These methylated lysines can then be recognized by HP-1, which then

binds through recognition with its chromodomain. Association of HP-1 with the DNA causes heterochromatinization and gene silencing. Thus this Su(var) protein, through its methylation of histone 3 plays a direct role in regulating gene expression (Lachner et al., 2001).

The Su(var) proteins contain "chromodomains" that are defined as a 50 amino acid residue found in chromosodomain proteins such as HP-1 and Pc (Polycomb genes). Little is known about the function of chromodomains, although mutation of *Drosophila* HP-1 suggests that it promotes an assembly of macromolecular complexes in chromatin (Jones et al., 2000). The recognition and binding of the chromodomain to methylated histones promotes the assembly of HP-1 at heterochromatin. The chromodomain of HP-1 is highly selective for methylated H3-lys9 in heterochromatin, but does not recognize or bind to H3 methylated at lysine 4, which is involved in activation (Bannister et al., 2001) (figure 1.6b). There are examples of proteins with two chromodomains that may recognize dimethylated histone tails. (for instance lysine 9 and lysine 27), which are situated in similar sequence motifs.

Bromodomains are a family of conserved protein modules that are in nearly all acetyltransferases. The bromodomain forms a left handed four-helix bundle with a conserved surface-accessible hydrophobic pocket. The hydrophobic pocket of the bromodomain binds to acetyl-lysine on histone tails (Zeng and Zhou, 2002). Bromodomains are associated with chromatin and present in many transcriptional regulators that have acetyltransferase activity. The $TAF_{II}250$ Su(var) protein has two bromodomains and preferentially binds with diacetylated histones (Jacobson et al., 2000). Proteins that contain up to six bromodomains may bind to different patterns of histone modifications and thereby affect gene expression (figure 1.6c). It may be that different proteins with different chromo or bromodomains affect gene regulation by inducing chromatin condensation by crosslinking (figure 1.6d).

Thus histone modifications influence chromatin structure and expression, and together may form another level of chromatin regulation. Histone variants may also play important roles in cell functioning, with each variant having a specific purpose in chromatin regulation.

1.6.5 Histone variants

Numerous histone variants occur, each with a specific purpose for chromatin structure and regulation (Table 1.1). For example CENP-A is a histone 3 variant found

Table 1.1: Histone variants

Histone variant	Difference from core histone	Localization	Function
CENP-A	Differs from H3 wth a non-canonical NH_2-terminal tail, divergent histone fold, and longer loop 1 region	Centromeres	Produces in a centromeric chromatin structure, essential for proper segregation
H1t	C-terminal end enriched in arginine. Lacks a H1 phosphorylation site	Pachytene and elongating spermatids	A role in spermatogenesis
H1°	Small, lysine rich and closely related Avian H5	Present in cells after differentiation	Strongly regulated with development, may associate with heterochromatin
mH2A	N-terminal third 64% identical to H2A. Other two thirds are a large non-histone region. The non-histone region is highly basic and interacts with DNA between neighbouring nucleosomes.	Heterochromatin and the X chromosome	Helps produce inactive chromatin
H2AZ	Sequences differences lead to interaction differences between the dimer and tetramer	Everywhere except for the inactive X chromosome and condensed heterochromatin	Unknown, may provide a specific chromatin state needed for protein binding
H2A-Bbd	Truncated C-terminal tail	Everywhere apart from the inactive X chromosome	Unknown, but as it localizes everywhere except for the inactive X chromosome, it may play a role in expression
H2AX	Contains a SQ (Serine, quanine) motif that is phosphorylated in DSB's	Double strand breaks	Necessary for localization of repair factors to the breakpoint

in centromeric DNA, and there are different testis specific histones as well as different variants of histone H2A. Several different histone variants have recently been discovered, and their functions and roles in chromatin packaging are being intensively studied.

1.6.5.1 Histone H1 variants

Many different variants of histone 1 can be found in somatic cells (eg. testis specific variant, H1t). One variant, H1°, is similar to the H5 found in the highly condensed and transcriptionally inactive erythrocyte nuclei of birds and fish (Doenecke and Tonjes, 1986). The concentration of H1° decreases in actively dividing cells suggesting a role in inactive chromatin states. However, growth, development, and fertility were unaffected in mouse homozygotes for a mutated H1° allele demonstrating that the histone variant is not essential for normal cell function (Sirotkin et al., 1995).

1.6.5.2 Histone H2A variant – Macro H2A

H2A has by far the largest family of variants, perhaps because the H2A-H2B dimer is more labile and will dissociate under lower-ionic strength conditions. A regulatory system based on dissociation of the H2A-H2B dimer has therefore evolved, as it requires less energy than dissociating the H3-H4 tetramer, which has a higher affinity for the nucleosomal DNA (reviewed by Ausio and Abbott, 2002).

Each protein has a Carboxyl tail and an amino (N) – terminal tail. Each amino acid has a carboxyl group (-COOH) linked to a side chain and an amino group (NH_2). Hydrogen bonds form between the carboxyl group of one amino acid and the amino group from another amino acid. The 5' end of the protein ends with a –COOH and is known as the C-terminus, whereas the 3' end of the protein ends with an -NH_2 and is known as the N-terminus (Chang, 1994). The histone carboxyl tails are found in the centre of the nucleosomes and therefore changes in the carboxyl tails of histone variants can affect nucleosome structure and stability. The N-terminal tales of histones are post-translationally modified affecting gene expression and chromatin structure.

MacroH2A (mH2A) is similar to H2A in its amino-N terminus, but differs in its other two thirds (Pehrson and Fried, 1992). Two different variants of histone macroH2A, mH2A1.1 and mH2A1.2, seem to have different roles, as mH2A1.2 levels increase during stem cell differentiation, whereas mH2A1.1 levels remain low (Pehrson et al., 1997). Perhaps the most interesting association of histone macroH2A is to the

inactive X chromosome of eutherian female cells (Costanzi and Pehrson, 1998), which occurs after the initiation of X inactivation (Mermoud et al., 1999). In *Xist* mutants, mH2A cannot localize to the inactive X chromosome, but X-inactivation still persists (Csankovszki et al., 1999). Therefore, mH2A may play a role in the maintenance of X-inactivation but is not essential for X-inactivation.

MacroH2A also localizes to centrosomes in embryonic stem cells and somatic cells (Mermoud et al., 2001), but to centromeres in mouse spermatocytes and round spermatids (Hoyer-Fender et al., 2000). The centrosome localization of mH2A in embryonic stem cells and somatic cells may reflect accumulation and degradation of mH2A (Chadwick et al., 2001, Mermoud et al., 2001), since components of a proteosome (that breaks down cellular proteins) are found at centrosomes. Indeed, when Chadwick and Willard (2002) inhibited the 20s proteosome, an increased amount of mH2A at the centrosome was observed.

The Macro chromatin body is formed by a high density of mH2A localizing to the inactive X chromosome, which is visualized cytogenetically with an anti rat mH2A antibody isolated from rabbits. The MCB forms on the inactive X chromosome after expression of *XIST* has taken place. The formation of the macro chromatin body (MCB) on the inactive X chromosome is present only at certain times of the cell cycle. Only 23% of G_1 cells had a MCB, compared to 72% of S phase cells (Chadwick and Willard, 2002). The timing of mH2A suggests that it is substituted for H2A on the inactive X chromosome at and around S phase. At metaphase the concentration of mH2A drops, and only four mH2A bands are present on the inactive X chromosome at Xp22, Xp11, Xq13, and Xq22-24. There is a clear overlap between staining at Xq22-24 with a dimethyl histone 3 lysine 4 antibody and an mH2A antibody, suggesting a boundary element that delimits the spread of mH2A. The four separate bands of mH2A have no discernable shared characteristics, but have been suggested to act as re-entry sites for *XIST* RNA (Chadwick and Willard, 2002). The incorporation of variants into the chromatin can therefore be very dynamic.

1.6.5.3 Histone H2A variant - H2AZ

Another important H2A variant of particular relevance in relation to this PhD thesis is H2AZ. H2AZ is a conserved histone that is found throughout eukaryotes, and is known as HTA3, H2AvD, Htz1p, and H2AZ.F in different organisms. H2AZ represents 5-10% of the H2A in a cell nucleus (West and Bonner, 1980) and is widely distributed throughout the nucleus showing no concentration in euchromatin or

heterochromatin in *Drosophila* (Leach et al., 2000). The function of this histone variant is still unknown. There are examples in which H2AZ is involved in transcriptional activation, or conversely in transcriptional silencing. In yeast, Htz1p (the yeast orthologue of H2AZ) is located at promoters (Santisteban et al., 2000). It is also found at the promoters of *GAL-10* genes in yeast and is essential for the recruitment of RNA polymerase II. Cells from which H2AZ is deleted are viable but grow slowly (Adam et al., 2001) and do not recruit RNA polymerase II to the *GAL-10* promoter. The c-terminal region of H2AZ also plays an important role in interactions with transcriptional complexes (Santisteban et al., 2000).

H2AZ may also inhibit gene expression in yeast. H2AZ is required for the silencing of the homologous mating region (HMR) locus by creating a specialized chromatin structure (Dhillon and Kamakaka, 2000). The HMR locus is silenced by binding *Sir1p* (silencer proteins) to the locus followed by interactions with *Sir2p, Sir3p* and *Sir4p*. It has been proposed that H2AZ leads to a more stable binding of the *Sir* complex to the nucleosomes (Dhillon and Kamakaka, 2000).

Although H2AZ is distributed throughout the cell, it is enriched in the nucleolus in metazoan cells (Allis et al., 1982). In yeast, H2AZ was distributed throughout the nucleus, with an enriched region of H2AZ co-localizing with Net1p (a nucleolar specific protein) at the nucleolus (Dhillon and Kamakaka, 2000).

Although H2AZ is not essential for viability in yeast, it was the first variant to be found to be essential for development in higher eukaryotes (Clarkson et al., 1999, Faast et al., 2001). Mice heterozygous for a knockout ($H2AZ^{+/-}$) were normal and fertile, but of the 52 offspring from intercrosses, none were homozygous for the mutant allele (Faast et al., 2001). Genotyping of embryos from intercrosses showed normal development of $H2AZ^{-/-}$ homozygotes until the 4.5 days postcoitum (d.p.c.). At days 5.5 and 6.5 d.p.c. $H2AZ^{-/-}$ mutants were underrepresented, obviously degenerated and were morphologically abnormal from the wild-type embryos and 7.5 d.p.c no $H2AZ^{-/-}$ embryos were detected, having reabsorbed (Faast et al., 2001).

Replacement of H2AZ with H2A residues at the C-terminal end of the protein proved lethal in *Drosophila* (Clarkson et al., 1999). H2AZ incorporation causes distinct changes in nucleosomes because of subtle destabilization of the interactions between the H2A-H2B dimer and the H3-H4 tetramer. Study of the crystal structure of H2AZ has demonstrated a manganese ion bound on the surface of the nucleosome. Similar

ions on other proteins promote protein-protein interactions and cause gene transcription (Suto et al., 2000).

H2AZ mimics closely the role of H4 acetylation in impeding the formation of highly condensed DNA (Fan et al., 2002). H2AZ also produces a localized chromatin folding via nucleosome-nucleosome interactions through the tail domains, promoting the regular positioning of nucleosomes (Fan et al., 2002). Therefore H2AZ can promote chromatin structures that either activate or inactivate genes. This is comparable to the dual role of H4, whose N-terminal tails also can either activate or repress transcription by their interaction with specific activators or repressors (Durrin et al., 1991, Kayne et al., 1988).

1.6.5.4 Other histone H2A variants

A new H2A variant, H2A-Bbd, has recently been discovered that seems to act oppositely to mH2A. An antibody to H2A-Bbd shows that H2A-Bbd is distributed throughout the interphase nucleus, except for the inactive X chromosome. At metaphase H2A-Bbd is also found on all chromosomes except the inactive X chromosome (Chadwick and Willard, 2001).

H2AX, another H2A variant, is involved in double-strand break repair, meiotic recombination, apoptotic digestion and class switch recombination during the development of immunoglobulin variability (reviewed by Ausio and Abbott, 2002). Co-localization of phosphorylated H2AX colocalizes with repair factors Rad50 and Rad51 at double strand breaks (Paull et al., 2000, Rogakou et al., 1998) is necessary for the localization of the repair factors to the breakpoint (Paull et al., 2000).

Histone variants are important in many cellular processes. Each variant has evolved for a specific purpose. However, the functions of macroH2A and H2AZ are still under debate. During this study the roles of macroH2A and H2AZ will be compared in mammalian meiosis to clarify their functions.

1.7 This study

It is now evident that there is a high level of nuclear organization within a cell. Chromosomes have non-random positions that are related to their gene content and activity, replication timing and is different between cell types. Individual genes also occupy non-random positions within the cell and chromosome territory that is related to their activity (reviewed by Francastel et al., 2000). Although it has been shown that

daughter cells have the same chromosome arrangement as the parental cells (Sun and Yokota, 1999), little is known about how this arrangement is maintained through mitosis and propagated. How this arrangement is passed on between generations is even more mysterious.

In this study, the arrangement of chromosomes in sperm was observed to see if an ordered chromosomal arrangement is passed onto the zygote. Since a non-random arrangement of chromosomes may set up at meiosis, the arrangement of chromosomes at meiosis was also studied.

Whether an ordered arrangement is merely an extension of the parental cell-daughter cell relationship or has some important function for setting up and initializing chromosome organization in the zygote was also addressed by examining to what extent the arrangement and the characteristics of chromatin was conserved.

In this study I observed chromosome organization in marsupials, monotremes and chickens to determine the generality of a non-random chromosome arrangement in sperm, and then compared the chromosome arrangement between two distantly related marsupials to determine if chromosomes have conserved positions.

The position of the X chromosome in marsupials and monotremes was of special interest because it may establish a connection between chromosome position and paternal X inactivation. Cross species painting was used to establish the conservation of the X chromosome between humans and monotremes. This was important in understanding the implications of a consistent apical position of the X chromosome in mammalian sperm.

For a specific chromosome organization to be established in sperm then the order must be determined during meiosis. Meiosis was studied by electron microscopy, and light microscopy. Using chromosome painting I observed the position of chromosomes during meiosis, and particularly the relationship of the X and Y chromosome. In eutherians, a synaptonemal complex forms between autosome pairs and between the pseudoautosomal regions on the X and Y chromosomes during meiosis, allowing pairing of the homologous chromosomes and recombination. In marsupials a synaptonemal complex forms between the autosomes but not between the sex chromosomes (Sharp, 1982). In this study I have used a specific SCP3 antibody to observe the marsupial synaptonemal complex, and electron microscopy to try and understand how the X and Y chromosome are kept together in marsupials.

Chromatin organization plays an important role in gene expression, affecting gene activity, and therefore function, of a cell. The mammalian X chromosome is characterized by a specific chromatin organization (hypoacetylation, mH2A and H2AZ localization). To try and understand how the paternal X chromosome is imprinted, chromatin organization was studied during marsupial meiosis.

The comparative studies should contribute to the understanding of chromosome and chromatin organization in mammalian cells.

CHAPTER 2: MATERIALS AND METHODS.

2.1 Animal and tissue collection

The species studied included the tammar wallaby (*Macropus eugenii*), the fat tailed dunnart (*Sminthopsis crassicaudata*), the southern hairy nosed wombat (*Lasiorhinus latifrons*), the platypus (*Ornithorhynchus anatinus*), the chicken (*Gallus domesticus*) and the mouse (*Mus musculus*).

Testis and/or sperm from *S. crassicaudata, M. eugenii,* and *L. latifrons* were collected by Doctor David Taggart (Melbourne University) and Professor Bill Breed (University of Adelaide). This material was collected under permit no. 100000572 (Victorian Department of Natural Resources and Development).

Dunnarts were killed by CO_2 saturation under permit number R.GC.04.01 from the ANU Animal Experimentation Ethics Committee. Mouse testis was collected under permit number LI2001204 and chicken testis under permit number LI2001204 from Environment ACT and protocol no. R.GC.02.00 from the ANU Animal Experimentation Ethics Committee. Animals were killed with 5ml of Sodium pentobarbitone injected into the pleural cavity.

2.2 Specimen preparation

2.2.1 Sperm preparation

Sperm were removed from the epididymis using a cut and squeeze method, and fixed in 10x volume of 3:1 methanol: acetic acid (AJAX). Briefly, testis was cut with a scalpel and the seminiferous tubules squeezed to remove spermatozoa. The sperm were washed three times in fixative, and stored at -20°C until required for study. Upon thawing, a drop of the sperm suspension was placed onto glass slides and allowed to air dry at room temperature. Preparations were then ready to be used for chromosome painting and fluorescence microscopy.

2.2.2 Testis preparation

2.2.2.1 Paraformaldehyde preparations

Paraformaldehyde preparations were used for immunofluorescence as this fixative preserves proteins. However, it does have a disadvantage in that it does not preserve DNA as well as methanol: acetic acid fixing.

Testis was mashed up in culture medium containing proteinase inhibitor (appendix 1) and centrifuged at 1200rpm for 10 minutes. The supernatant was taken out and replaced with 0.5M sucrose (BDH). Slides were prepared by placing 75µl of 1% paraformaldehyde (pH 8) (Sigma) onto the slide, then adding one drop of the cell suspension in sucrose. Slides were left in a moisture chamber (a container filled with paper and moistened with distilled water) for 2 hours, and then washed in water ready for use. Slides could be kept for only 2 days, after which the proteins had degraded so they would no longer be recognized by the antibodies.

2.2.2.2 Methanol: acetic acid preparations

To prepare meiotic cells for chromosome painting, testis was mashed and immersed in a hypotonic solution (0.075M KCl) (AJAX) for 30 minutes at 37°C to swell the cells and allow the chromosomes to separate. The tissue was then immersed three times in fixative (3:1, methanol: acetic acid) and left at -20°C until required. The cell suspension was dropped onto slides washed in acetone and dried overnight. Preparations were washed through an ethanol (EtOH) series (3 mins in 70% EtOH, 3 mins in 90% EtOH and 3mins in 100% EtOH) and stored at -80°C until needed.

2.2.3 Testis sectioning

Fresh testis tissue was sectioned for protein immunofluorescence. Sectioning through seminiferous tubules allows the observation of the progression of meiotic cell stages. Fresh testis from mouse and dunnart was placed in 4% paraformaldehyde (pH 7.4) (appendix 1) and left to fix for 2-3 hours. Testis was then washed in phosphate buffered saline (PBS) for 2 minutes, then put through an ethanol series, into xylene (solvent) according to the protocol in table 2.1 and into paraffin (Oxford). They were embedded under a vacuum to ensure paraffin was perfused throughout the testis. Sections were fixed to a wooden block and left to set overnight at room temperature.

The sections were cut at 5µm on a microtome and flattened out by placing in a water bath containing a small amount of 70% ethanol. The sections were then floated

Table 2.1: Paraffin embedding

Solution	**Vacuum**	**Time (minutes)**	**Temperature (°C)**
50% ETOH	-	20	RT
70% ETOH	-	20	RT
90% ETOH	-	20	RT
95% ETOH	-	20	RT
100% ETOH	-	20	RT
100% ETOH	-	20	RT
100%ETOH	-	20	RT
Xylene	-	20	RT
Xylene	-	20	RT
Paraffin	+	20	58
Paraffin	+	20	58
Paraffin	+	20	58

RT = room temperature

ETOH = ethanol

onto acetone-washed slides, which were then left at 37°C overnight. Slides were stored at room temperature (RT) until needed for use.

2.2.4 Cell culture, origin of cell lines

Cells were cultured in Dulbecco's modification of minimal medium (DME) (GibcoBRL, Melbourne) supplemented with 10% fetal calf serum (GibcoBRL, Melbourne) and grown by Mrs. Iole Barbieri (Latrobe University) and Mrs. Pat Miethke (The Australian National University). Several different animal cell cultures were used during this study, which have been summarized in table 2.2.

2.2.5 Mitotic cell preparation

Cells were treated with 50ng/ml – 75ng/ml Colcemid (Roche, Mannheim) for 2 - 4 hours to arrest cells at metaphase. Culture medium was removed from the flask and the cells were rinsed with 4ml PBS to remove all of the culture medium as it inhibits trypsin. PBS was removed and trypsin (GibcoBRL, Melbourne) was added to the flasks to detach cells. Cells were collected by centrifugation at 1200g for 10 minutes, the supernatant removed and the cell pellet resuspended in 2ml of hypotonic warmed to 37°C. Fixative was then added up to a volume of 10ml and the suspension spun at 1200g for 10 minutes, after which the supernatant was removed and cells resuspended in 10ml of fixative. This was repeated three times. The pellet was resuspended in 0.5-1.5ml of fixative (depending on cell number), and the preparation dropped onto cleaned slides. Chromosome paints were then hybridized to these cells, to test the quality of the chromosome paint.

2.3 Chromosome paints

2.3.1 Flow-sorting chromosomes

Chromosomes were flow-sorted by a fluorescence activated chromosome sorter (FACS) machine in Professor Malcolm Ferguson-Smith's laboratory Cambridge, UK. Metaphase chromosomes were stained with two different fluorescent dyes, one of which binds to AT rich DNA, and the second to GC rich DNA. The cells are lysed and the chromosomes are fixed. The whole chromosomes were then placed into a suspension, which was converted into a concentrated spray, each droplet containing one

Table 2.2: Cell lines.

ANIMAL	TISSUE
Homo sapien	Blood
Mouse species hybrid (B3) *Mus musculus* x *Mus caroli*	Embryo
Sminthopsis crassicaudata	Skin
Sminthopsis douglasi	Male = Body wall Female = Skin from front limb
Macropus eugenii	Male = ear Female = ear
Vombatus ursinus	Skin
Ornthithorynchus anatinus	Toe webbing
Gallus domesticus	Cornea

chromosome. The spray is then passed through a laser beam, which is excited by fluorescence, each chromosome produces its own fluorescence signature, which is recognized and directed into a specific tube (reviewed by Griffiths et al., 1996).

2.3.2 DOP-PCR

DNA from the isolated chromosomes was amplified by two DOP-PCR reactions. The first reaction was performed to obtain templates (see appendix 1), which were then submitted to a second PCR, this time with biotin-16-dUTP or digoxigenin-11-dUTP (see appendix 1). The products from the second DOP-PCR were then used as probes. DOP-PCR is a general amplification of the whole genome or in this case a single whole chromosome.

The DOP-PCR reaction randomly amplifies the whole chromosome. The second reaction uses biotin or digoxigenin, which is incorporated randomly throughout the amplified chromosome in place of thymidine. These "labeled" chromosomes can then be visualized after hybridization by using antibodies conjugated with fluorescent dyes that recognize either biotin or digoxigenin.

2.3.3 DNA electrophoresis

0.8% agarose gel was made by adding 0.4g of low melting agarose (GibcoBRL, Gaithersburg USA) to 50ml of 0.5 x TBE (appendix 1). The solution was heated in a microwave oven for 1 minute to allow the agarose to dissolve. Ethidium bromide (BDH, Kilsyth, Australia) was then added.

After the gel was set, 50ng/µl of the ΦX174 marker (appendix 1) and 7µl of the PCR product (5µl of DNA along with 2µl of Bromophenol blue (appendix 1) were loaded in the gel and allowed to run for 30 minutes at 100 volts and 25 milliamperes in 0.5 x TBE. A photograph of the gel was taken in the dark on an UV box in order to estimate the concentrations and the size of the DNA.

2.3.4 Primed *in situ* labeling (PRINS)

Metaphase preparations were made using methanol: acetic acid fixation (section 2.2), and air dried. Slides were denatured in 70% formamide/2xSSC at 72°C for three

minutes, washed through an ice cold ethanol series and air dried. Formamide was used to lower the denaturing temperature of the DNA.

50µl of a reaction mix (appendix 1) was added to each slide and the drop was covered with a coverslip, and sealed with rubber cement. *In situ* PCR amplification was undertaken on slides using a program consisting of 10 minutes annealing time at 65°C and 30 minutes at 72°C. The slide was immersed in 500mM NaCL/50mM EDTA, pH8 at 70°C for 1 minute. Slides were washed 2x3minutes in 2xSSC/0.5% Tween 20, stained with DAPI, and mounted in Vectorshield under a coverslip.

2.4 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization was used in this study to localize whole chromosome probes ("painting") or telomere-specific probes. Hybridization with a single paint involved using biotin labeled paints. Double painting used one chromosome paint labeled with biotin (yellow or green) and a different chromosome paint labeled with digoxigenin (red), allowing two chromosomes to be distinguished in one cell.

2.4.1 Probe treatment

Whole chromosome probes were pretreated with suppressor (blocking) DNA. Suppressor DNA blocks repetitive units within the probe that may bind all over the genome. A ratio of 1:50 or higher of probe to suppressor DNA was used. As there are multiple copies of the repeats present in the blocking DNA and also in the probe, these repeats should bind reasonably quickly, once the DNA is allowed to re-anneal. The unique sequences required in the probe to hybridize to a specific chromosome do not anneal to identical sequences as quickly as there are less of the sequences in the suppressor DNA. As a result after a certain amount of time all repetitive sequences should be annealed and unique sequences will still be single stranded. This can then be hybridized to the slide where the single stranded unique sequences will hybridize to the specific chromosome and the double stranded repeats will not hybridize and can be washed from the slide at a later stage.

2.4.1.1 Suppressor DNA

Boiled DNA or sonicated DNA (suppressor DNA) was used to block repeat sequences in the probe from binding non-specifically and producing background signals.

A frozen piece of tissue from the animal being studied (eg. *M. eugenii*) was crushed with a mortar and pestle, and the ground tissue added to 15ml of extraction buffer (appendix 1). Proteinase K (Roche) was then added at a final concentration of 100ug/ml and the suspension left in a 50°C water bath for 3 hours to help breakdown the tissue and proteins. The suspension was then spun at 1600g for 10 minutes and the supernatant was transferred to a clean tube.

Proteins were removed from the DNA by two extractions with phenol (equilibrated to pH8 (Sambrook et al., 1989), followed by one phenol/chloroform/isoamylalcohol (25:24:1) extraction. Phenol was added to the supernatant containing the DNA and left to mix overnight before centrifuging at 3000g until the organic and aqueous phases were separated. The aqueous phase was collected and an equal volume of phenol: isoamyl-choloform (1:1) was added to separate the proteins from the DNA. The mixture was centrifuged at 3000g, and then the aqueous phase (which contained the DNA) was moved to a clean tube and the same procedure carried out until the interface between the two layers was clean.

The clean supernatant was collected and ice cold ethanol (2.5 x volume of the supernatant) was added to precipitate the DNA. The DNA was then collected with a glass rod, or pelleted by centrifugation. The precipitated DNA was resuspended in 500-1000µl TE (appendix 1) or distilled water and incubated at 37°C for 30 minutes along with 200ul of 10mg/ml Rnase (appendix 1) to degrade RNA in the sample.

The extracted genomic DNA was then boiled for 50 minutes to shear it into 200-500bps fragments. The DNA was then run on a 0.8% agarose gel and photographed to check the size of the boiled DNA against the λ marker (appendix 1). Suppressor DNA is essential to block out repetitive sequences in the chromosome paint.

2.4.1.2 Pretreatment of the probe

400ng of labeled probe was added to an Eppendorf tube along with blocking DNA to a ratio of 1:50 (probe: blocking DNA). A 100-fold excess of 3M NaOAc (appendix 1) was added to blocking DNA and probe DNA, then 2.5 volumes of ice cold ethanol was added. The mixture was left to precipitate at -20°C for 2 hours or overnight, then

centrifuged at 4°C for 15-20 minutes at 13,000g to pellet the DNA. The ethanol was removed and the pellet air-dried. 10-15µl of a hybridization mixture (appendix 1) was added and the solution incubated at 37°C for 30 minutes.

The DNA was denatured at 80-90°C for 6 minutes to separate the strands, and then pre-annealed at 37°C for 20 minutes to allow the repeat sequences to hybridize to the suppressor DNA. In some experiments, the denatured probe was left at 37°C for 45 minutes to pre-anneal its own repetitive sequences, in which case suppressor DNA was not required. The probe was then ready to be added to the treated slide so that the unique single stranded sequences could hybridized to the DNA of the sample.

2.4.2 Specimen pretreatment

Specimens must be pretreated before they can be hybridized with the chromosome paint. The chromosome paint will bind to RNA within the cell nucleus producing non-specific background signals, and therefore RNA must be removed from the specimen. Specimens may also need a pepsin digestion to breakdown proteins and cytoplasm surrounding the nucleus as these may block the paint from hybridizing to the DNA. The specimens must then be denatured so the DNA is single stranded and is able to bind to the chromosome paint.

2.4.2.1 RNase digestion

A solution of 0.1mg/ml RNase was placed on the slides and covered by a large coverslip. The slide was then incubated for 90 minutes in a 2xSSC moisture chamber at 37°C to degrade all the RNAs. Slides were then washed four times in 2xSSC (appendix 1) for 2 minutes, and then either dehydrated in an ethanol series (3 minutes each in 70%, 90% and 100% ethanol at room temperature) or digested with pepsin to increase the accessibility of the DNA to the probe.

2.4.2.2 Pepsin digestion

The slides were incubated in a Coplin jar with 0.05% pepsin (Sigma) in distilled water at a pH of 2.3 at 37°C for 10 minutes. The slides were then washed twice in PBS for 5 minutes, once in PBS/50mM $MgCl_2$ (appendix 1) for 5 minutes, 1% formaldehyde in PBS/50mM $MgCl_2$ for 10 minutes, PBS for 5 minutes and dehydrated in an ethanol row.

2.4.2.3 DNA denaturation

After the RNase and pepsin treatments, the slides were placed in a Coplin jar containing 70% formamide (AJAX)/2xSSC (appendix 1) at 70-75°C. Slides with testis and sperm samples were left in a Coplin jar for 150 seconds, and slides with somatic cells were left in a Coplin jar for 130 seconds. After denaturation the slides were placed straight into an ice cold ethanol series for 3 minutes at each step. Slides were allowed to dry completely before the denatured probe was added to the slides, as ethanol can inhibit hybridization of the probe.

2.4.2.4 Hybridization and washing

The denatured probe was placed on the slide at the area of interest and a coverslip (22mmx22mm) placed on it and sealed with rubber cement. Slides were incubated at 37°C in a 2 x SSC moisture chamber for 48 hours for a hybridization of a homologous probe, or for 5 days for a cross-species hybridization. This provided enough time for the probe to hybridize to the target DNA. Cross-species hybridizations required longer as they have fewer homologous sequences to hybridize.

The hybridized slides were washed in three baths of 50% formamide/2xSSC (pH 7), one bath of 2xSSC (pH7) at 42°C for 5 minutes and 0.1 x SSC at 60°C for 5 minutes. Slides were then left in blocking solution (appendix 1) for 45 minutes.

2.4.3 Probe detection

Chromosome paints are visualized by using antibodies against biotin or digoxigenin that are conjugated with fluorescent dyes. The slides are washed to remove antibody that has not bound, the DNA stained with DAPI allowing the cell nucleus to be visualized and mounted with an anti-fade solution. An anti-fade solution is important so that the fluorescent dyes do not bleach and disappear when excited through fluorescence.

2.4.3.1 Antibody preparation

Single, double or triple antibody layers were used to amplify the signal. All antibodies used in this study were diluted in blocking solution (appendix 1). Sources and characteristics of antibodies are shown in table 2.3.

2.4.3.2 Immunodetection of painting probes

Slides were incubated with 200µl primary antibody (100ul of each primary antibody for a double paint) at 37°C for 45 minutes in a 2xSSC moisture chamber in the

Table 2.3: Antibodies for chromosome painting

Antibody	Company	Dilution of 1mg/ml	Animal source
Anti biotin	Vector	3:500	Goat
Anti goat FITC	Vector	1:100	Rabbit
Anti dig	Vector	3:500	Mouse
Anti mouse TRITC	Vector	1:100	Goat
Anti dig cy3	Jackson	1:200	Mouse
Anti sheep FITC	Jackson	1:100	Rabbit
Anti rabbit TRITC	Jackson	1:100	Goat
Anti Avidin biotin	Vector	3:500	Goat
Anti Avidin FITC	Vector	1:100	N/A
Anti sheep FITC	Jackson	1:100	donkey

dark. Slides were then washed twice in washing solution (4 x SSC/0.2%Tween 20) at room temperature for 5 minutes. 200µl of the second antibody (100ul of each secondary antibody for a double paint) was then added to the slides (if needed), which were placed in a 2 x SSC moisture chamber for another 45 minutes at 37°C in the dark, and again washed in washing solution. The above procedure was repeated if a third antibody was used.

To stain the chromosomes, slides were placed in a 4' 6-diamidino-2-phenyl-indole (DAPI) solution (7µl of .1mg/ml DAPI in 50ml of distilled water) for 2 minutes and washed twice in distilled water. Vectorshield (Vector) mounting medium was then placed on the slide followed by a coverslip.

2.4.3.3 Detection of telomeric probes

Slides were prepared and denatured as described in section 2.4. A telomeric probe $(CCCTAA)_7$ end labeled with a cy3 molecule was obtained from Gene Works (Adelaide, Aus). 1µg of probe was placed in 20µl hybridization mixture (appendix 1), incubated at 80°C for 6 minutes and put onto the slide. A coverslip was added and sealed with rubber cement. The slide was incubated in a moisture chamber at 37°C for 24 hours. The slides then went through 3x5 minute washes in 50%formamide/2xSSC at 40°C. Slides were DAPI stained and mounted with Vectorshield and a coverslip.

2.4.3.4 Fluorescence microscopy and imaging

Slides were analysed with a Zeiss Axioplan 2 fluorescent microscope and the images captured with a SPOT RT monochrome camera from Diagnostic Instruments incorporated (Sterling Heights, USA) using Zeiss filter sets 02 (excitation 360nm, emission 450nm onwards, used for DAPI), 10 (excitation 450-490nnm, emission 515-565nm, used for FITC) and 15 (excitation 546nm, emission 590nm, used for TRTIC). The pictures were produced with IPlab (Fairfax, USA) and Adobe Photoshop 5.5 and Adobe Illustrator 10 were used to produce the final images.

2.5 Electron microscopy

2.5.1 Fixation

Samples were collected under permit number R.CG.02.00 from the ANU Animal Ethics Committee from wallabies killed under permit number R.DN.60.01. The

wallabies were sedated with Ketamine (20mg/kg of body weight) and Rompun (2mg/kg of body weight). After 10 minutes, an overdose (10 mls) of 5% Pentothal was injected intravenously l. 4% paraformaldehyde was then perfused (pumped via the blood vessels) through the tammar wallaby to fix testis for electron microscopy. Small 3mm x 3mm square pieces were cut from the centre of the testis and washed three times in PBS.

The pieces of tissue were then post-fixed in 1% OsO_4 (osmium tetroxide) in 25mM Phosphate buffer, pH 6.8-7.2, for 1-2 hours. These pieces were then washed three times for 5 minutes each in 25mM phosphate buffer.

2.5.2 Embedding

The samples were taken twice through a graded series of 30 minute ethanol washes (10%, 25%, 50%, 70%, 95% and 100%). The sections could be left overnight once they had reached the 70% ethanol bath.

The samples then underwent a resin infiltration procedure, in which they were transferred through 2:1, 1:1 and 1:2 mixtures of LR White Resin (Sigma) in ethanol. The samples were left at each stage for 2-3 hours, and then transferred into 100% resin for 1-2 hours. The samples were then transferred into fresh resin and left overnight, before transferring the samples and resin into gelatin molds. The resin was polymerized (hardened) at 60°C for 24 hours.

2.5.3 Sectioning

The gelatin mold was removed and secured in a holder. A razor was then used to produce a 1mm x 1mm surface on the resin block. An ultramicrotome (Reichert, Ultracuts) was used to cut the samples. A glass knife was made and tape used to make a reservoir of water on the outside edge of the glass knife. The resin was then manually cut until the sectioning reached the samples. Once this occurred, the ultramicrotome was switched to cut the sections automatically. Sections of 60-90nm were cut and placed on copper or nickel grids, which were stained with 6% uranyl acid for 20 minutes, rinsed with water, left to dry and stored at 4°C until needed for study.

2.5.4 Electron microscope.

Samples were observed on a transmission electron microscope (Hitachi H7100FA TEM, 1995). The TEM microscope has a double diffusion pump vacuum

system, electronic image and used a LaB_6 cathode. Pictures were captured by a 22 plate large format camera, and the negatives were developed by Mrs. Lily Cheng, who also produced some scanning electron microscope pictures that have been included within this thesis.

2.6 Protein localization

Histone proteins were detected during marsupial and mouse meiotic and somatic cells through antibodies raised against the particular protein (table 2.4).

2.6.1 Protein localization in mitotic cells

Cells were grown in 10ml flasks with culture medium by Mrs Pat Miethke. Colcemid was added to the flask for a final concentration of 200ng/ml. Cells were then incubated further for 2hrs (for tammar) or 4hrs (for platypus), until many loosely adhering (rounded up) mitotic cells were observed. Trypsin was added for 10–15 seconds, and the detached cells (mostly mitotic cells) were spun down at 1500rpm for 10 minutes. Cells were then washed twice in PBS and swelled in 0.075M KCl for 10 minutes at room temperature. Cells were counted on a haemocytometer and diluted to a concentration of 2-4 x 10^4 cells/ml. Acetone washed slides were placed in a steal frame that has a plastic tube attached. 200µl of the cell suspension was added to the plastic tube and spun down onto the slides in the Shandon cytospin at 1000rpm for 10 minutes. As the centrifuge spins, the cells within the tube are smashed against the slide spreading the mitotic cells.

Slides were placed in KCM buffer (appendix 1) for 8 minutes, then in blocking solution (KCM buffer + 1% BSA) for 10 minutes. 100µl of antibody diluted in KCM buffer + 1% BSA (for dilution see table 3) was added to the slide and incubated for 1hr at 37°C in a moisture chamber. Slides were washed 2 x 10 minutes in KCM buffer. The secondary antibody was placed onto the slides, which were incubated for 45 minutes at 37°C, then washed 2 x 10 minutes in KCM buffer. Cells were fixed by soaking slides in 4% paraformaldehyde for 10 minutes, then were washed once in PBS and counterstained with DAPI (section 2.4.3.2).

Table 2.4: Protein antibodies

Antibody	Company/person	Dilution	Animal made in
SCP3	Terry Ashley	1:250	Goat
MacroH2A	John Pehrson	1:100	Rabbit
Acetyl H4 (lys 5)	Upstate technologies	1:100	Rabbit
Acetyl H4 (lys 8)	Upstate technologies	1:100	Rabbit
Acetyl H4 (lys12)	Upstate technologies	1:100	Rabbit
Acetyl H4 (lys 16)	Upstate technologies	1:100	Rabbit
H2AZ	David Tremethick	1:250	Sheep
Methyl H3 (lys 9)	Upstate technologies	1:100	Rabbit
Phosph H3	Upstate technologies	1:100	Rabbit
Acetyl H4	Upstate technologies	1:100	Rabbit
Acetyl H3	Upstate technologies	1:100	Rabbit
αHP-1	David Tremethick	1:100	Mouse
InCENP	David Tremethick	1:100	Rabbit

2.6.2 Protein localization in interphase cells

Tammar wallaby fibroblast cells were grown directly on slides in a slide culture chamber in a $C0_2$ incubator. The culture vessel was removed then slides were placed in 4% paraformaldehyde for 10 minutes and then washed in PBS. Cells were permeabilized by incubating slides in 0.1% SDS/1%BSA in PBS for 15 minutes, then placing in blocking solution (1%BSA in PBS) for 45 minutes at 37°C. Antibodies against the protein being studied were diluted in antibody solution and added to the slides and incubated overnight at 4°C. Slides were then washed 4-5 times in cold PBS. A secondary antibody was added and incubated at 37°C for 45 minutes. Slides were then washed 4-5 times in cold PBS and counterstained with DAPI (as previously described).

The first antibody, made in sheep, bound to the protein within the permeabilized cell. A second antibody that was made against the source of the primary antibody (in this case anti-sheep) was then added. The secondary antibody was conjugated with a fluorochrome, and could be detected. A fluorescence microscope with the DAPI, FITC and TRITC filters can then excite the fluorochrome making the fluorescent dye visible. Different fluorochromes can be used in conjunction if they are excited at different wavelengths.

2.6.3 Protein localization in meiotic cells

Testis preparations and sections (see sections 2.2.2 and 2.2.3) were placed in 0.1% SDS/ 1% BSA in PBS for 15 minutes to permeabilize the cell membranes. Slides were then placed in a blocking solution (1%BSA in PBS) for 45 minutes at 37°C. The antibody, diluted in antibody solution, was then added to the slides, which were incubated overnight at 4°C. Slides were then washed 4-5 times in cold PBS. A secondary antibody was added and incubated at 37°C for 45 minutes. Slides were then washed 4-5 times in cold PBS and counterstained with DAPI (as described in section 2.4.6).

CHAPTER 3: CHROMOSOME ARRANGEMENT IN SPERM

3.1 Introduction

Chromosome painting and confocal microscopy of interphase mammalian nuclei have shown that chromosomes occupy distinct non-random territories that depend on their gene concentration and transcriptional activity (reviewed by Cremer and Cremer, 2001). Genes themselves also occupy non-random positions within nuclei (Parreira et al., 1997), with active genes at the surface of their chromosome territories or facing interchromatin domains and inactive genes in the interior (Chevret et al., 2000). In somatic cells, chromosome position in daughter cells is inherited from the parental cell (Sun and Yokota, 1999).

Such a somatically heritable pattern may reflect functional significance. It is particularly important, therefore, to discover whether chromosome arrangement is transmitted between generations. This makes the study of chromosome arrangement in germ cells crucial. In the zygote, nuclear organization needs to be set up, therefore imprints and nuclear organization carried from the sperm may be critical in establishing the chromosome organization within the zygote.

Chromosome arrangement in sperm has been studied over decades, but the low resolution afforded by conventional cytological techniques and the variety of material, have led to inconsistent results. This study is one of only a few to study chromosome arrangement in sperm using modern chromosome painting techniques to determine whether there is a non-random chromosome arrangement in sperm, to study its conservation and characteristics, in order to deduce what function it may play in setting up the nuclear organization of the zygote.

3.1.1 Studying chromosome positions in sperm

Sperm have many features that make them easy material for the study of chromosome arrangement, compared to somatic cell types such as fibroblasts and cancer cells. Thus, sperm were used as a model for chromosome organization from as early as the 1940s.

The shape and morphology of sperm shows remarkable diversity throughout the

animal kingdom (reviewed by Poccia, 1986) (figure 3.1). Asymmetrical spermheads and the position of the acrosome and tail enable researchers to orientate the position of a chromosome within the cell. In some animals, the shape of the spermhead allows chromosome positions to be determined easily. For example chicken, some insects and monotreme sperm have long fibrilliar spermheads, facilitating accurate determination of chromosome positions.

It is much more difficult to study chromosome arrangement in interphase cells, since spherical cells and nuclei provide no landmarks to orientate the cell or provide specific reference points to position chromosomes. A further complication is that chromosome position may change during the cell cycle as chromosomes go through replication, condensation and movement to the metaphase plate (reviewed by Mitchison and Salmon, 2001), all of which may alter chromosome position, or gene position within a chromosome territory. In an asynchronous population a chromosome position may therefore appear to be random or non-random but be a result of cell replication and division and have no functional significance.

There are some difficulties in studying chromosome arrangement in sperm due to the small size of the spermhead, and the extremely tightly packaged DNA. This packaging is accomplished by replacing the histones with protamines, which are cross-linked in eutherian sperm. The condensed DNA makes it hard for chromosome paints to access and hybridize to the sperm DNA.

3.1.2 Arrangement of chromosomes in sperm

Chromosome arrangement in insect sperm was studied in the 1940s, and from early on there were conflicting results using different techniques on different species (table 3.1). Feulgen staining was used to observe chromosome positions in the fibrillar sperm of Iceryine coccids, which have only two chromosomes (n=2) (*Steatococcus tuberculatus, Echinicerya anomala,* and *Icerya purchasi*). The 2 chromosomes had a non-random end-to-end association with chromosome 2 in front of chromosome 1 (Hughes-Schrader, 1946). Irradiation of the needle-like sperm of the cave cricket (*Ceuthophilus nigricans*) with polarized ultraviolet light demonstrated that the coiled chromosomes are tandemly arranged one after another lengthwise in the spermhead (Inoue and Sato, 1962). Grasshoppers (*Romalea microptera*) were injected with H^3-thymidine and the thin elongated sperm nuclei were collected 60 days after injection.

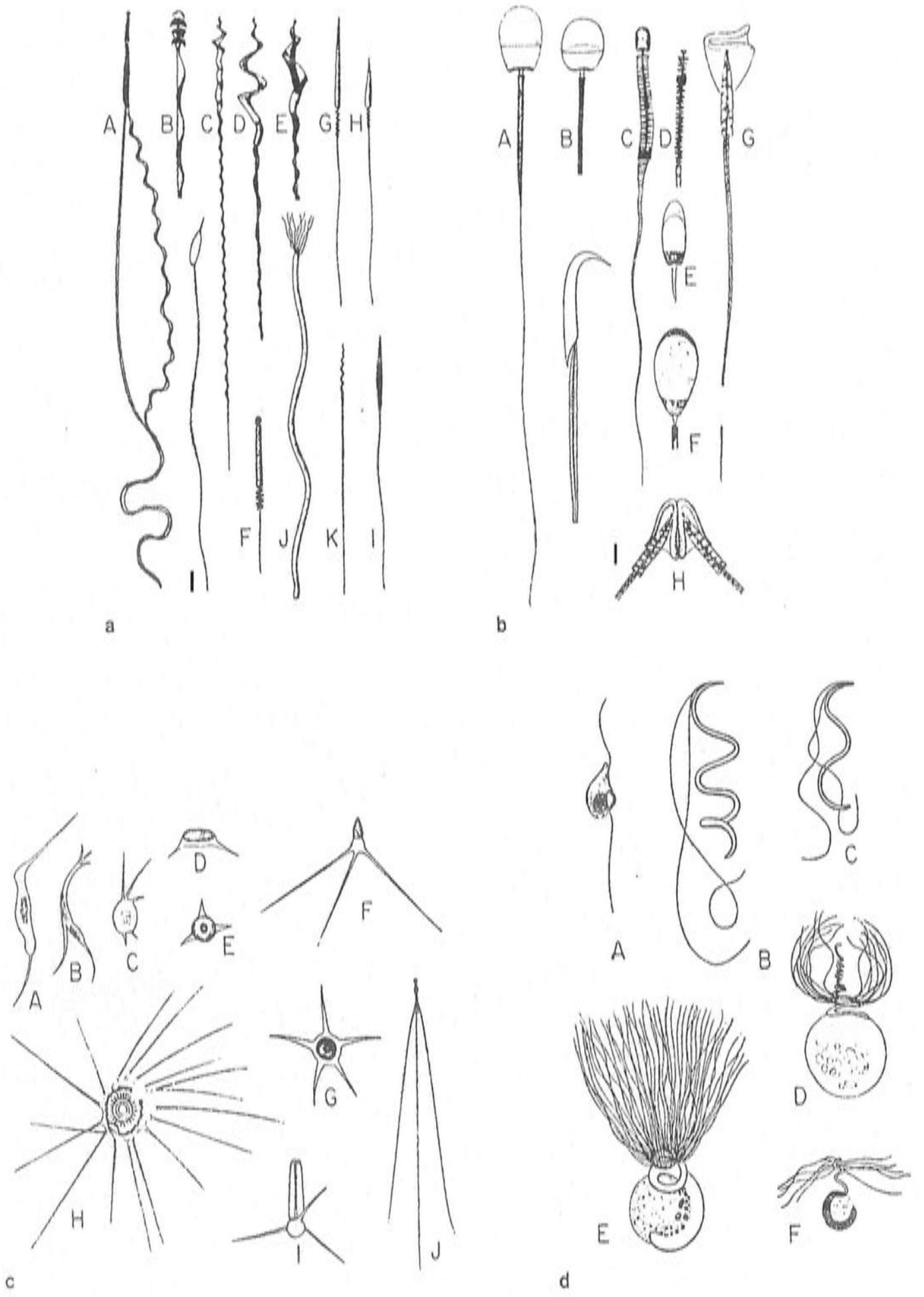


Figure 3.1: Spermatozoa morphology.
(a) From animals: **A** beetle (*Corpis*), **B** insect (*Calathus*), **C** bird (*Phyllopneuste*), **D** bird (*Muscicapa*), **E** bullfinch, **F** gull (*Larus*), **G-I** *Tadorna*, **J,K** snail (*Paludina*), and **L** snake (*Coluber*).
(b) From mammals: **A,B** badger, **C-E** bat (*Vesperugo, Rhinolophus*), **F** pig, **G** opossum, **H** opossum, double spermatozoon, and **I** rat
(c) Unusual spermatozoa: **A-C** crustacean (*Polyphemus*), **D,E** crab (*Dromia*), **F** *Ethusia*, **G** *Maja*, **H** *Inachus*, **I** lobster (*Homarous*), and **J** crab (*Porcellana*)
(d) From plants: **A** alga (*Fucus*), **B** liverwort (*Pellia*), **C** moss (*Sphagnum*), **D** *Marsilia*, **E** fern (*Angiopteris*), and **F** fern (*Phegopteris*). Reproduced from Poccia (1986).

Table 3.1: Chromosome arrangement in animal sperm

SPECIES	METHOD	CHROMOSOME POSITIONING NON-RANDOM/RANDOM	REFERENCE
INSECT			
Iceryine coccid	Feulgen staining	Non-random	Hughes-Schrader et al. 1946
Cave cricket	Polarized ultraviolet light	Non-random	Inoue & Sato 1962
Grasshoppers	Autoradiography of early and late replication	Random, tandem arrangement[a]	Taylor 1964
PLATHELMINTHES			
Planarians	FISH: telomeric and rDNA probes	Non-Random	Joffe et al. 1998
AMPHIBIANS			
Salamanders	Autoradiography of centromeric heterochromatin	Random	Macgregor & Walker 1973
Anura	Q- and C-banding	Random	Schmid 1979
BIRDS			
Chicken	C-banding	Tandem arrangement	Dressler & Schmid 1976
	FISH: telomeric, repetative and chromosome 6 probes	Random	Solovei et al. 1998
MAMMALS			
Monotremes:			
Echidna and Platypus	FISH: telomeric, radioactive in situ for rDNA and gene specific probes	Non-random, tandem arrangement	Watson et al. 1996
Eutherians:			
Hamsters	Feulgen staining	Non-random, tandem arrangement	Douglas 1965
Rat	FISH: centromeric probes and chromosome painting	Centromeres, telomeres Non-random	Meyer-Ficca et al. 1998
Human	FISH: centromeric and telomeric probes Chromosome painting	Non-random	Zalensky et al. 1995; Luetjens al.1999; Hazzouri et al. 2000

[a]Tandem arrangement: Chromsomes aligned one after another down the length of the sperm head

The tritium was injected at three different times, to represent all DNA, represent early replicating DNA where the X is not labeled and to represent late replicating DNA where only the X chromosome was labeled. This produced three differently labeled spermheads, in the first type all chromosomes were labeled, in the second type the X chromosome was labeled and very little else (late replicating), and in the third type everything labeled apart from the X chromosome (early replicating). The author suggested that the pattern of tritium grains in sperm demonstrated a tandem arrangement of chromosomes. The distribution of tritium grains in sperm where the X had been labeled was random (Taylor, 1964).

Different techniques have also been used to study chromosome position in amphibian sperm. Salamander sperm (n=13) (*Plethodon cinereus*) was hybridized with H^3 RNA complementary to centromeric satellite heterochromatin. The signals were always found at the posterior of the sperm, indicating a non-random position of centromeres. The chromosomes form U shapes in the sperm, with their centromeres clustered at the rear of the spermhead (Macgregor and Walker, 1973). Heterochromatin position was examined by Q- and C- banding in eight species of frog sperm. This demonstrated that specific Q- bright (AT rich) heterochromatin did not occupy any specific position within the sperm of the eight frog species (Schmid, 1979).

More recently probes to telomeric, rDNA, repetitive and specific genes have been used to observe chromosome positions in sperm by *in situ* hybridization. In the elongated sperm of planarians *Dendrocoelum lacteum* (n=7) and *Polycelis tenuis* (n=7), five to eight clusters of telomeres were found in a seemingly random pattern in mature sperm (Joffe et al., 1998). However, the 28S rDNA genes clustered together in sperm, providing some evidence for non-random arrangement of chromosomes in planarian sperm. Also, 5S rDNA genes (located in subcentromeric zones on two or three pairs of chromosomes) were clustered at the posterior of the spermhead.

In mature chicken sperm *Gallus domesticus* (n=39), telomeric probes produced 5–10 signals, with no distinct pattern (Solovei et al., 1998). Hybridization of a specific Z chromosome repetitive sequence (CZMR) again showed a random distribution of the signal. A probe to a specific gene (Gd/6), found on the long arm of chromosome 6, also occupied variable positions in chicken sperm. The position of all three probes show that chromosome arrangement is inconsistent in chicken sperm. The inconsistent arrangement of chromosomes in chicken sperm contrasts with the non-random position of chromosomes observed in chicken fibroblasts and neurons, in which

microchromosomes were preferentially located towards the interior, and macro-chromosomes tended towards the periphery of the cell nucleus (Habermann et al., 2001). At mitosis, a stringent radial arrangement of chromosomes was found, with early-replicating DNA (mostly microchromosomes) towards the interior, and late replicating DNA towards the periphery. Given the non-random arrangement of chromosomes in chicken somatic cells, it seems extraordinary that chromosomes should be randomly arranged in sperm.

Telomeric, alpha satellite and unique sequence probes have been used to study nuclear organization in monotreme and eutherian mammals. In platypus (2n=52) and echidna (male, 2n=63) *in situ* hybridization with telomere-specific oligonucleotides produced a consistent pattern of 26 and 31 bands respectively along the fibrillar spermhead. This was interpreted as a tandem, non-overlapping arrangement, with telomeres juxtaposed (Watson et al., 1996). Radioactive *in situ* hybridization for rDNA and unique sequence probes (genes PGK, F8, G6PD and DMD) demonstrated non-random positions of chromosomes in monotreme sperm.

Tandem arrangement of chromosomes was also observed by Feulgen staining in Chinese hamster sperm (2n=22) (*Cricetulus barabensis*) in which the 11 chromosomal elements are tandemly aligned within the sperm that had been pretreated with EDTA. However, this observation was not observed in untreated sperm (Douglas, 1965). In decondensed human sperm, *in situ* hybridization with telomeric probes demonstrated that telomeres are non-randomly positioned at the periphery of the nucleus (Zalensky et al., 1995). Binding with the centromeric protein (CENP-A) demonstrated that centromeres cluster at the centre of human sperm. In rat sperm (n=42) (*Rattus norvegicus*), pericentromeric regions of nine chromosomes were detected with a satellite centromeric specific probe (Meyer-Ficca et al., 1998). Like the human centromeres, rat centromeres clustered together, running down the centre of the spermhead. Telomeric probes also hybridized to the periphery of the spermhead in rat. Thus, in all mammalian species studied, some form of nuclear organization has been observed.

Overall however, the results are inconsistent, with examples of both random and non-random distribution of chromosomes in sperm of insects, planaria, amphibians and birds. Many of the techniques used have insufficient specificity and resolution. For instance, Feulgen staining, staining of heterochromatin, telomeric probes, replication timing, radioactive *in situ* hybridization and repetitive probes all can give only partial

information on position of chromosomes in a cell. Furthermore, with the possible exception of some repetitive probes, none of the methods allow specific chromosomes to be identified.

Chromosome painting offers a great advantage, as the whole chromosome can be visualized and specific chromosomes identified. Chromosome paints are by far the most specific and sensitive way in which to study chromosome positions, especially when this is combined with the use of 3-D confocal microscopy (reviewed by Cremer and Cremer, 2001).

Chromosome paints have recently been used to establish that the positions of chromosomes 2 and 12 in rat sperm are non-random. The rod-shaped rat chromosome 2 territory was found in the medial region extending either to the anterior or posterior end of the sperm. The smaller chromosome 12 territory was found preferentially in the medial region of the spermhead.

Chromosome painting was also used to observe the position of three chromosomes in human sperm. The X chromosome was found to lie non-randomly in the anterior half of the sperm nucleus, whereas chromosome 13 seemed to have a random position within the sperm nucleus (Hazzouri et al., 2000b). A separate experiment using a chromosome X paint and a chromosome 18 paint, demonstrated the anterior position of the X chromosome and the posterior position of chromosome 18 in human sperm (Luetjens et al., 1999).

Overall, then, chromosome arrangement seems to be non-random in mammalian sperm, but not in bird sperm.

3.1.3 Aims of this study

In this study I aimed to discover whether specific chromosome arrangements are transmitted between generations in animal sperm. Firstly, the organization of chromosomes in marsupials and monotremes was studied in order to test the generality of a non-random chromosome arrangement in mammalian sperm. Secondly, the arrangement of chromosomes was compared between two distantly related marsupials, the dunnart and the wombat (which share a conserved karyotype). A conserved arrangement in these species would suggest that the arrangement is functionally significant. The X chromosome position was compared between the three mammalian groups to understand if its position in sperm may play an important role in paternal X-inactivation. Lastly, this study challenged the surprising finding that chromosome

arrangement is random in chicken sperm.

3.2 Results

3.2.1 Chromosome arrangement in marsupial sperm

3.2.1.1 Marsupial sperm morphology

During this study I found wide differences in hybridization frequencies with different types of sperm and also with different fixation techniques. Normal fixed methanol: acetic acid preparations undergo different levels of decondensation during the fixation and FISH procedure, allowing probes to hybridize. This does not happen to sperm that previously have been fixed with a formalin fixative. Formalin based fixatives fix proteins, allowing the protamines to hold the DNA tightly condensed. There may also be differential condensation between the different animals studied, which explains the different success levels of telomeric hybridizations seen in marsupial sperm.

The morphologies of the dunnart and wombat sperm provided advantages in this study of the chromosome arrangement in the spermhead. The dunnart has an arrow shaped head with the tail protruding from the medial region of the ventral surface (figure 3.2a, 3.3a). The acrosome lies along the dorsal surface of the spermhead.

The spermhead of the wombat is hook-shaped and the acrosome is positioned on the ventral surface (figure 3.2b and 3.3b). Variations of wombat sperm morphology were observed which corresponded with seven of the eight variations described previously (Breed et al., 2001) (figure 3.4). The different wombat sperm morphologies raise the possibility of differential viability and differential success rates at fertilization. The variation in sperm morphology also raises the possibility of different chromosome positions within each morphologically distinct spermhead.

Marsupial protamines lack disulfide bonds between cysteine residues and so marsupial sperm are less stable than eutherian sperm. This means that sperm could be decondensed during FISH, and chromosome arrangement could be disrupted. However, I observed that the X chromosome position was the same in condensed and decondensed sperm (figure 3.5), and concluded that the decondensation is not enough to affect chromosome position.

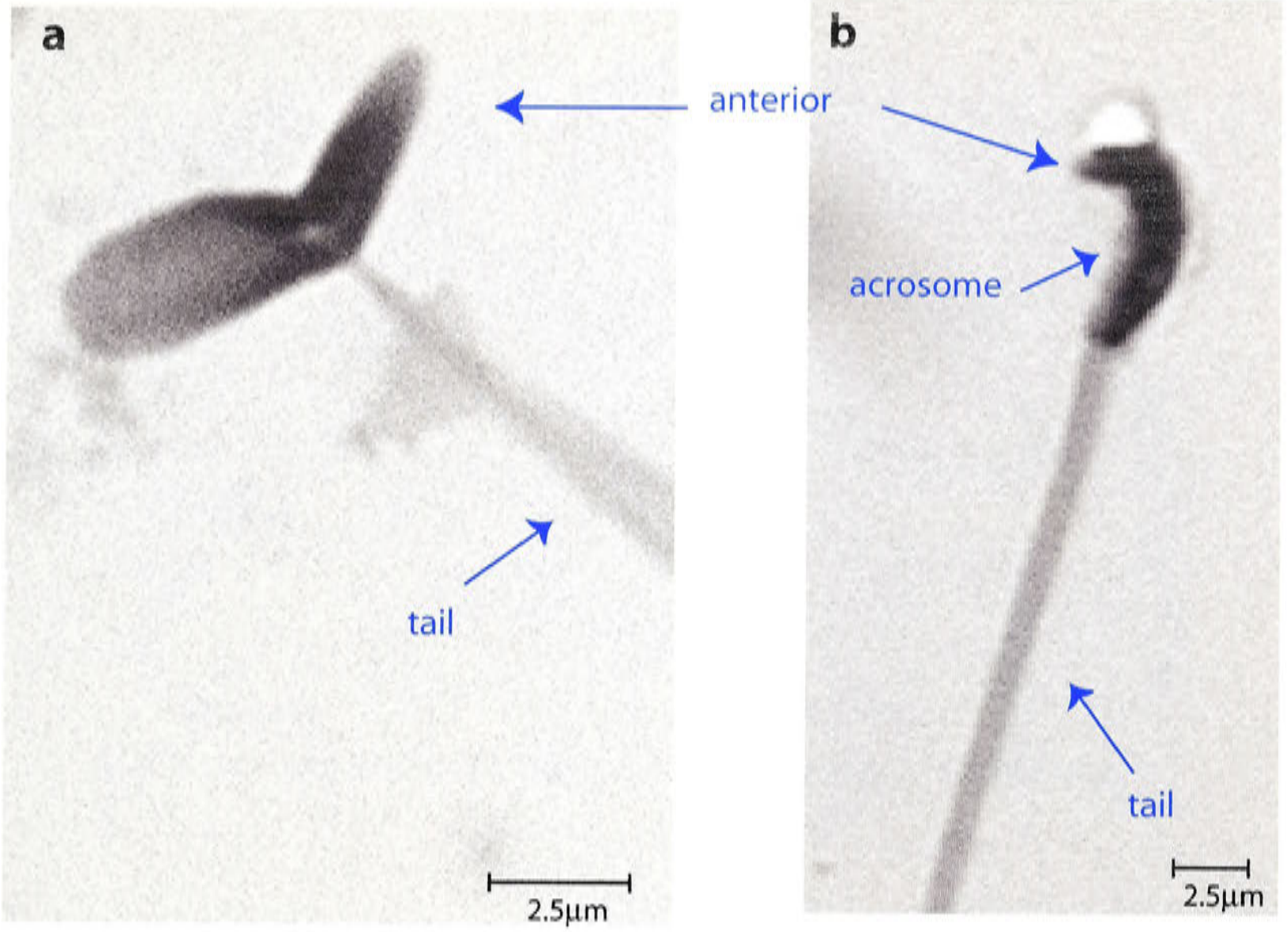


Figure 3.2: Light microscope images. Giemsa stained sperm from marsupials, monotremes and chickens. (a) Immature dunnart sperm, in which the head forms a T-shape with the tail. (b) Wombat sperm (one of 8 variants in sperm morphology). (c) Platypus sperm with pointed anterior region, covered by the acrosome. (d) Chicken sperm showing that theanterior end is slightly more rounded. However, anterior and posterior ends could not be reliably distinguished after FISH.

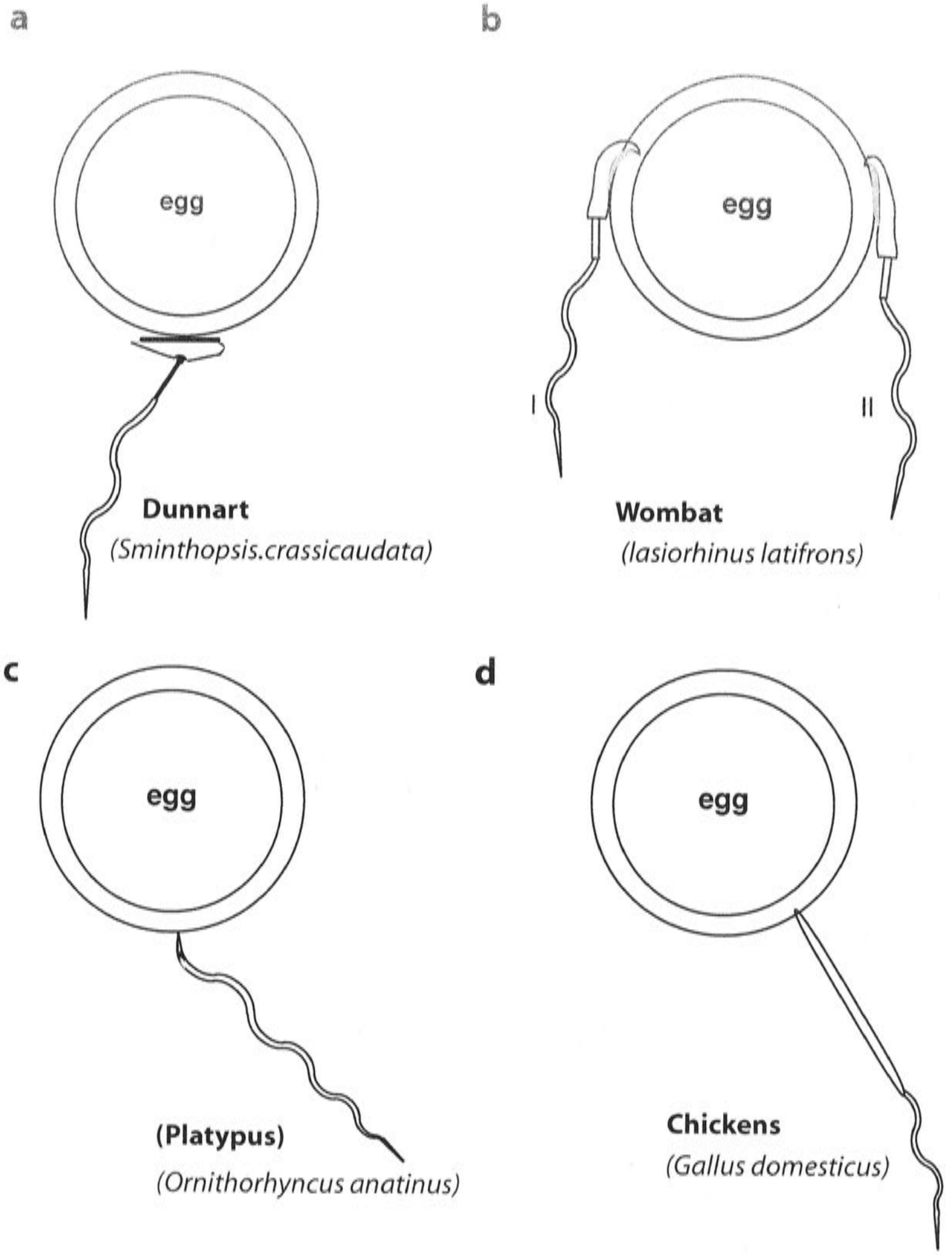
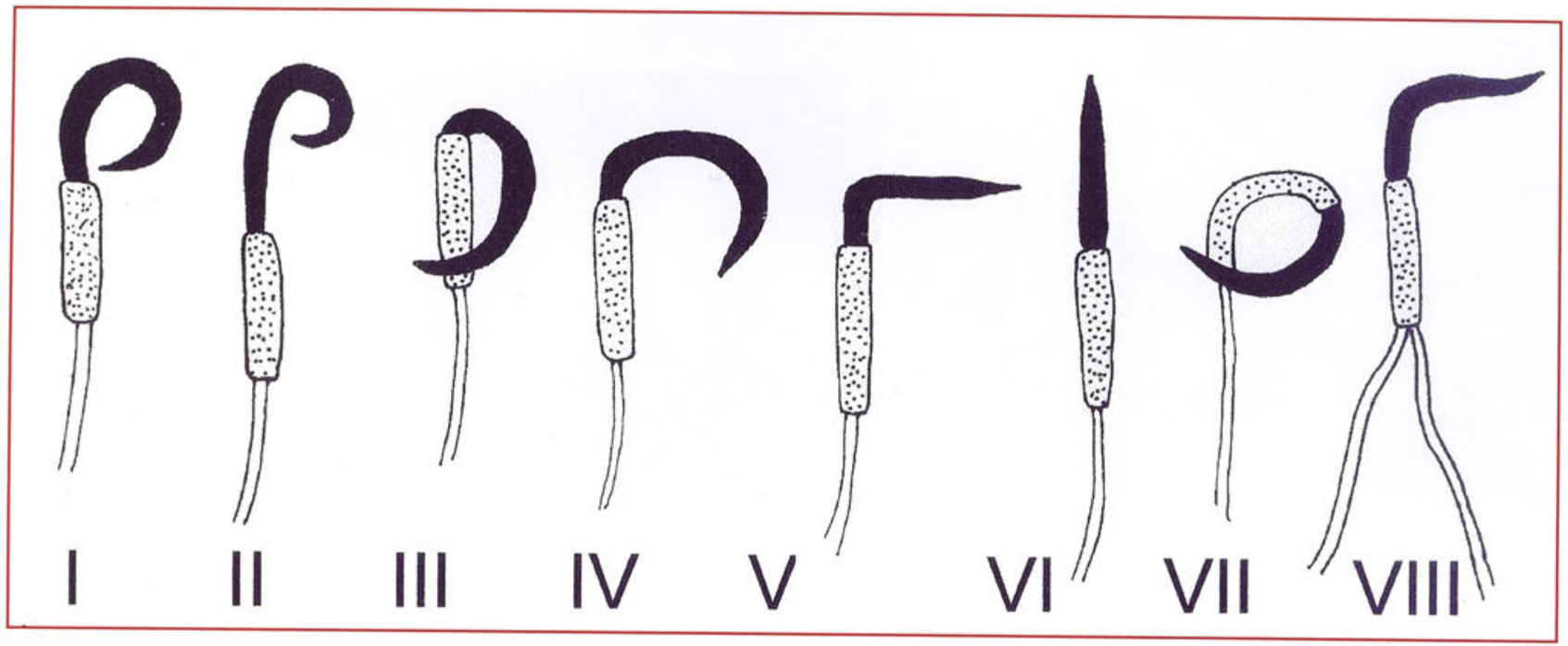


Figure 3.3: Sperm-egg fusion in marsupials, monotremes and birds. (a) Sperm-egg fusion observed in the dunnart occurs asthe spermhead turns on a hinge and forms a T-shape. (b) Presumed sperm-egg fusion in wombat. Two of many possible sperm-egg interactions are shown. (I) The spermhead may hook into the egg allowing the acrosome to come into contact with the egg, (II), sperm head may be flexible and bend to allow the acrosome to come into contact with the egg. (c) Platypus sperm -egg fusion. (d) Chicken sperm-egg fusion.

a



b

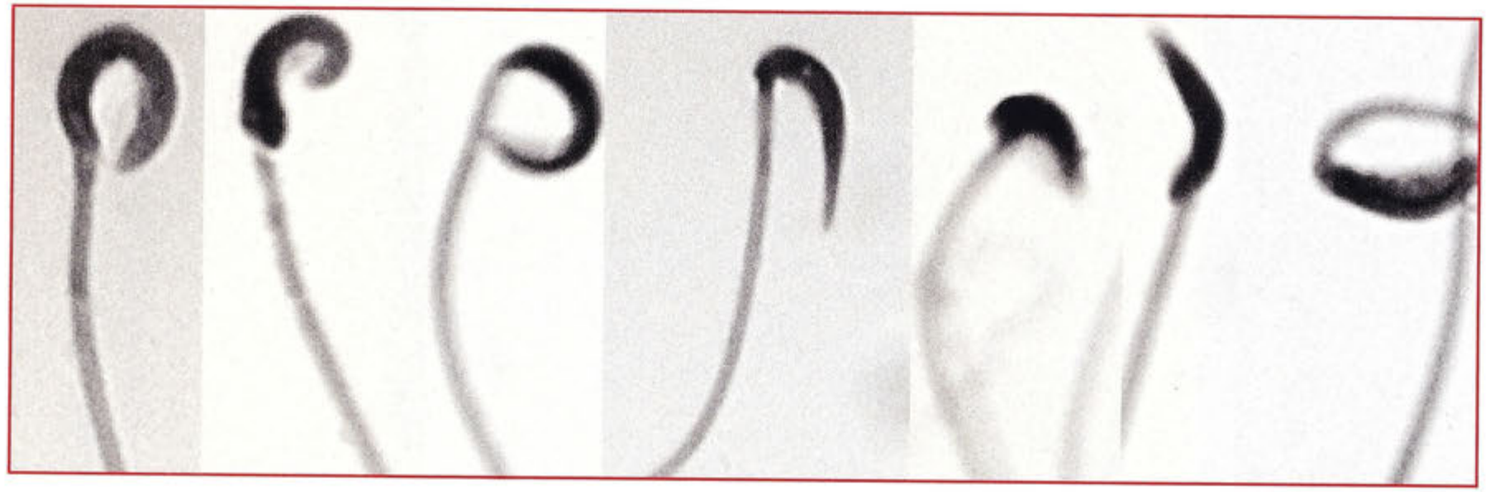


Figure 3.4: Sperm morphology in the Southern hairy nosed wombat. Wombat sperm has eight recognizable morphologies, I - VIII (a), of which seven were observed in this study (b).

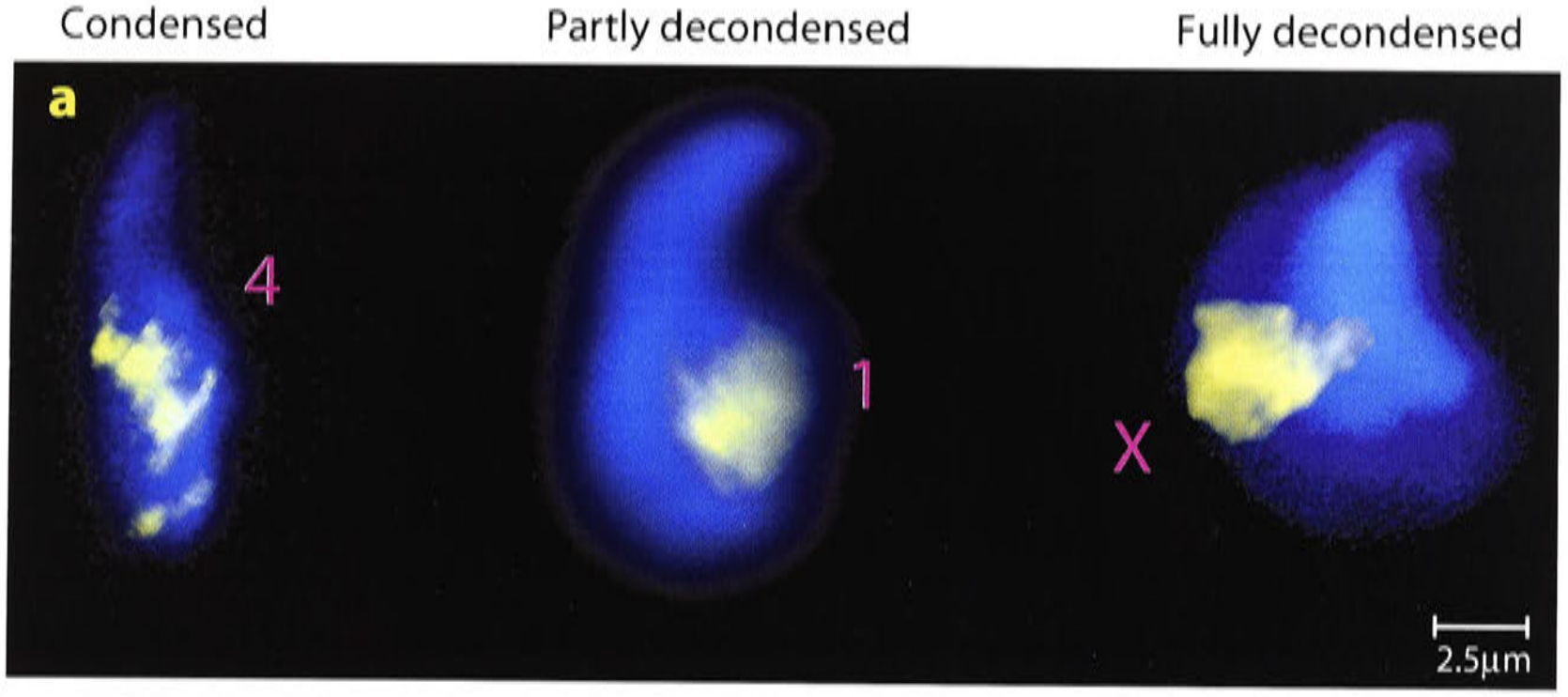


Figure 3.5: Sperm decondensation during FISH. Differential condensation of the sperm was observed (a). However, this did not affect chromosome position (b). The numbers/letters refer to the chromosome paint used.

3.2.1.2 Marsupial chromosome- specific sorting and paints

Flow-sorted chromosomes were prepared in the laboratory of Professor Malcolm Ferguson-Smith. Chromosomes were classified by their size and base content into discrete peaks on fluorescence activated chromosome sorter (FACS), then were physically separated into chromosome-specific samples. DNA from each peak had first to be painted to metaphase spreads to see which chromosome each corresponds to. In wombat sorts, chromosome 1 corresponded to peak 2, chromosome 2 corresponded to peak 5, chromosome 3 corresponded to peak 3, chromosome 4 corresponded to peak 1, chromosome 5 corresponded to peak 7, and the X chromosome corresponded to peak 4 (figure 3.6). One peak representing chromosome 6 (by a process of elimination), yielded DNA unable to produce a signal, conversely, the X chromosome produced the best quality signal (figure 3.6). Evidently the quality of peaks can vary between different flow-sorted chromosomes.

Noticeably each paint also hybridized to heterochromatic regions of the X chromosome. A high concentration of suppression DNA was used for each paint to block these heterochromatic signals. The chromosome-specific probes were applied singly or in pairs to preparations of sperm from the homologous species.

3.2.1.3 Chromosome arrangement in wombat sperm

Positions of signals were recorded in at least 15 sperm for all chromosomes except 6 (which provided an unacceptably weak signal). Double labeling with two colors permitted determination of the relative positions of two chromosomes (figure 3.7). Problems of interpretation resulted from the cross-hybridization of paints to the heterochromatin part of the X chromosome, so double painting was extremely important. All double painting experiments involved an autosome and the X chromosome to enable differentiation of the X chromosome signal from the autosome signal. The co-localization of both paints to the heterochromatic region enabled the autosome, the X chromosome and the heterochromatic region of the X chromosome to be recognized. For example, if the autosome signal were green and the X chromosome signal red, the X chromosome heterochromatin would be yellow (as both paints contain this sequence).

In wombat sperm the bulk of the DNA was found in the medial-posterior region of the spermhead. Only chromosome 3 was found at the narrow anterior of the spermhead. Chromosomes 4, 5, and X were found preferentially in the medial region of the spermhead, and chromosomes 1 and 2 were positioned at the posterior region. This

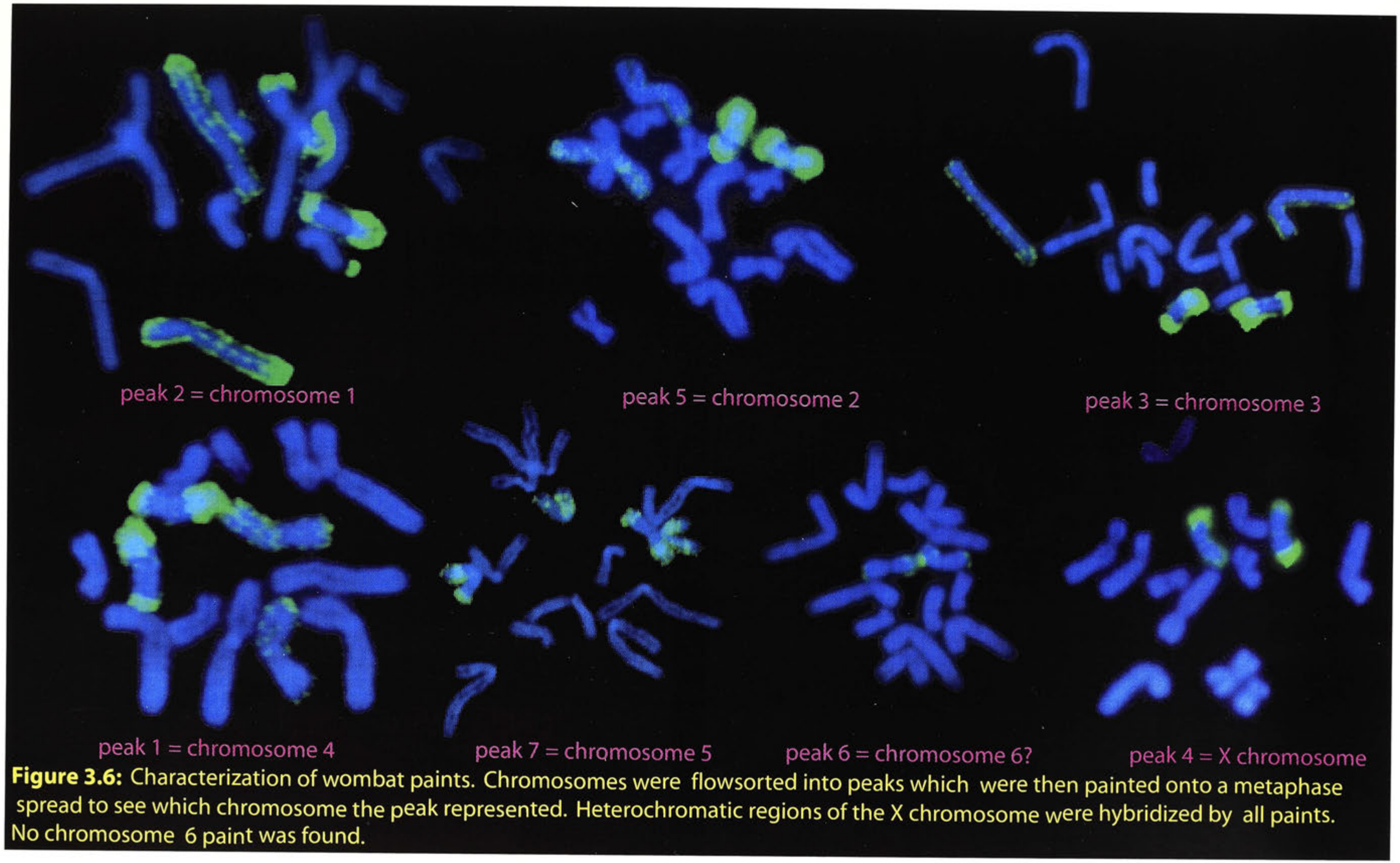
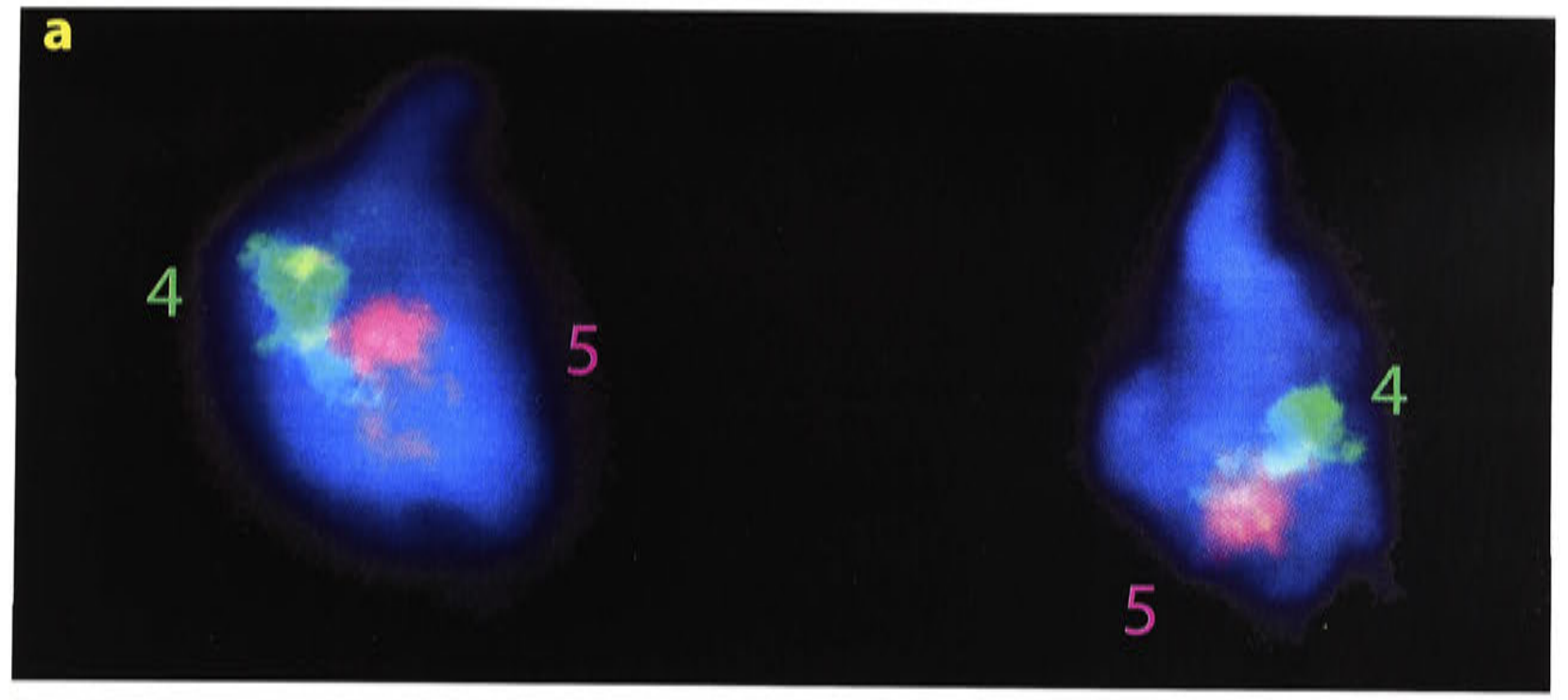
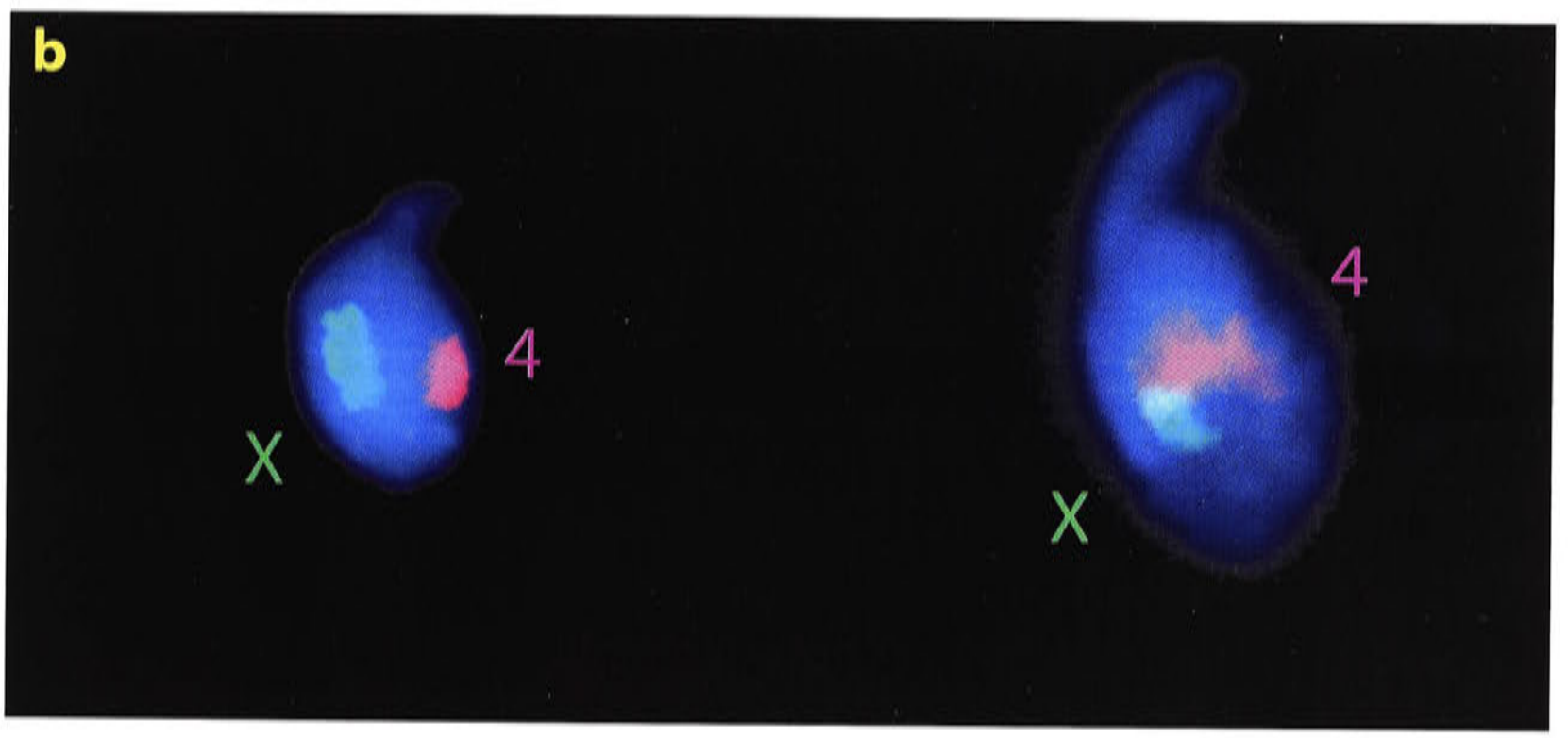


Figure 3.6: Characterization of wombat paints. Chromosomes were flowsorted into peaks which were then painted onto a metaphase spread to see which chromosome the peak represented. Heterochromatic regions of the X chromosome were hybridized by all paints. No chromosome 6 paint was found.





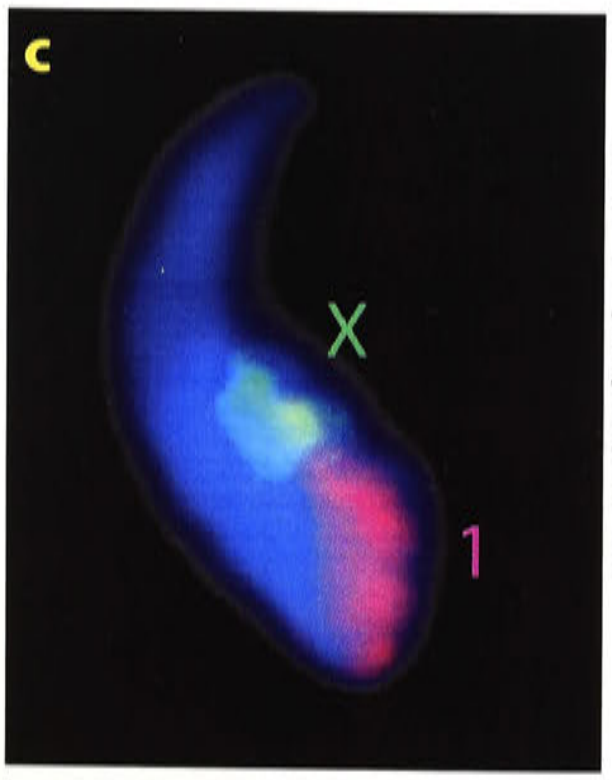


Figure 3.7: Double painting in wombat sperm. Numbers and letters represent chromosome paint, with green biotin labeled and red digoxigenin labeled. (a) Chromosome 4 and 5 were located in a similar position with chromosome 4 slightly anterior to chromosome 5. (b) X chromosome or chromosome 4 were both medial with chromosome 4 anterior to the X chromosome in most sperm. (c) The X chromosome had a medial position, and chromosome 1 a posterior position.

provided a non-random chromosome arrangement from the anterior to the posterior of 3 – 4 – 5 – X – 1 and 2 (figure 3.8, table 3.2).

After DAPI staining it was not possible to differentiate the different wombat sperm morphologies. However, the low variability of signal position in the spermhead suggests no difference in chromosome arrangement between the different morphologies. Slight variations in the positions were observed, but these were attributed to the misinterpretation of signals in the spermhead as two-dimensional analysis sometimes made it difficult to interpret spermhead orientation.

3.2.1.4 Conserved chromosome arrangement in marsupial sperm

The dunnart and wombat both share the ancestral marsupial karyotype of 2n=14 (section 1.1.3.4). Comparative chromosome painting has shown that these two karyotypes are extremely similar, with only slight intrachromosomal rearrangements. In this study I have compared the chromosome arrangement I observed in wombat sperm with the non-random chromosome arrangement I previously observed in the dunnart (figure 3.8 table 3.3) (Greaves et al., 2001). If the chromosome arrangement in mammalian sperm has an important functional role for the zygote, the arrangement observed in dunnart and wombat would be expected to be conserved.

It is easy to recognize comparable positions in the hook-shaped wombat and the arrow-shaped dunnart sperm. In both species, chromosome 3 was observed at the anterior of the sperm nucleus, chromosomes 4, 5 and X occupied the medial region and chromosomes 1 and 2 occupied the posterior region of the spermhead (figure 3.9,). Chromosome 6 could not be compared between the two species. Thus the arrangement of chromosomes in marsupial sperm has been conserved across at least 50 million years of evolution (figure 3.10) (Greaves et al., 2003).

3.2.1.5 Telomeres in marsupial sperm

To better understand the overall organization of chromosomes within the spermhead, telomeric probes were used to study telomeric localization in sperm. Preliminary work showed telomeric signals positioned at the periphery of the nucleus and running down the length of the dunnart spermhead (Greaves et al., 2001). This suggested models of centromeric and telomeric positioning within mammals (figure 3.11). I therefore analysed telomeric positions in marsupial sperm using a 42 bp long oligonucleotide, $(ccctaa)_7$, end labeled with cy3, hybridized firstly to metaphase spreads for each animal and then to sperm (figures 3.12-3.14).

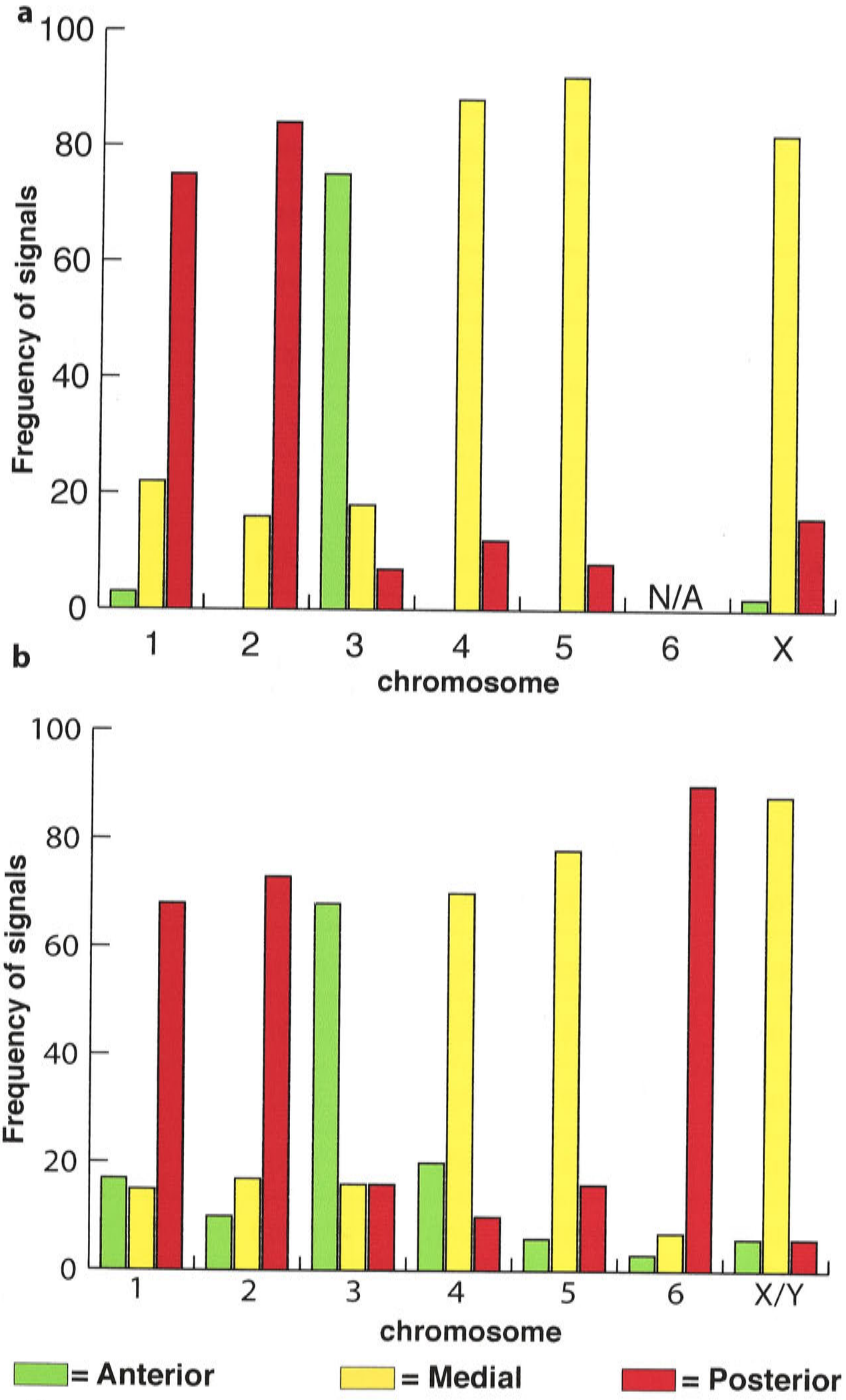


Figure 3.8: Position of signal wombat sperm (a) and dunnart sperm (b)

Table 3.2: Position of signal in wombat sperm.

Chromosome	Numbers (%) of sperm with signal			
	Anterior	Medial	Posterior	Total
1	1 (3%)	8 (22%)	**28 (75%)**	37
2	0 (0%)	8 (16%)	**42 (84%)**	50
3	**21 (75%)**	5 (18%)	2 (7%)	28
4	0 (0%)	**42 (88%)**	6 (12%)	48
5	0 (0%)	**12 (92%)**	1 (8%)	13
6	N/A	N/A	N/A	N/A
X	1 (2%)	**41 (82%)**	8 (16%)	50

Table 3.3: Position of signal in dunnart sperm.

Chromosome	Numbers (%) of sperm with signal			
	Anterior	Medial	Posterior	Total
1	11 (17%)	10 (15%)	**45 (68%)**	66
2	3 (10%)	5 (17%)	**22 (73%)**	30
3	**34 (68%)**	8 (16%)	8 (16%)	50
4	12 (20%)	**41 (70%)**	6 (10%)	59
5	3 (6%)	**40 (78%)**	8 (16%)	51
6	1 (3%)	2 (7%)	**27 (90%)**	30
X/Y	4 (6%)	**57 (88%)**	4 (6%)	65

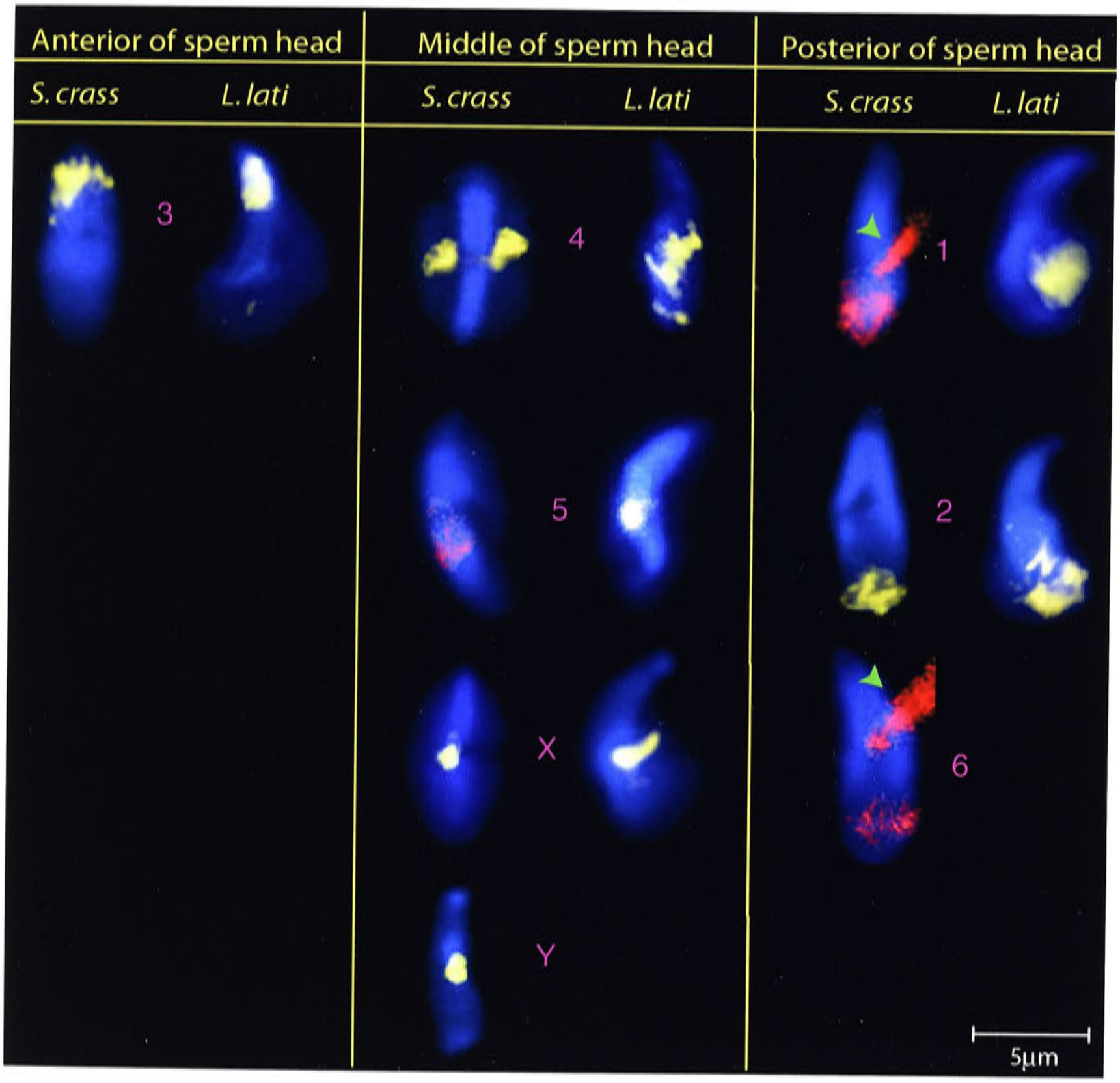


Figure 3.9: Comparison of chromosomes between the dunnart and the wombat. Biotin labeled chromosomes are in yellow and digoxigenin labeled chromosomes are in red. Sperm painted for chromosomes 1 and 6 in the dunnart also have non-specific antibody binding to the tail (➤).

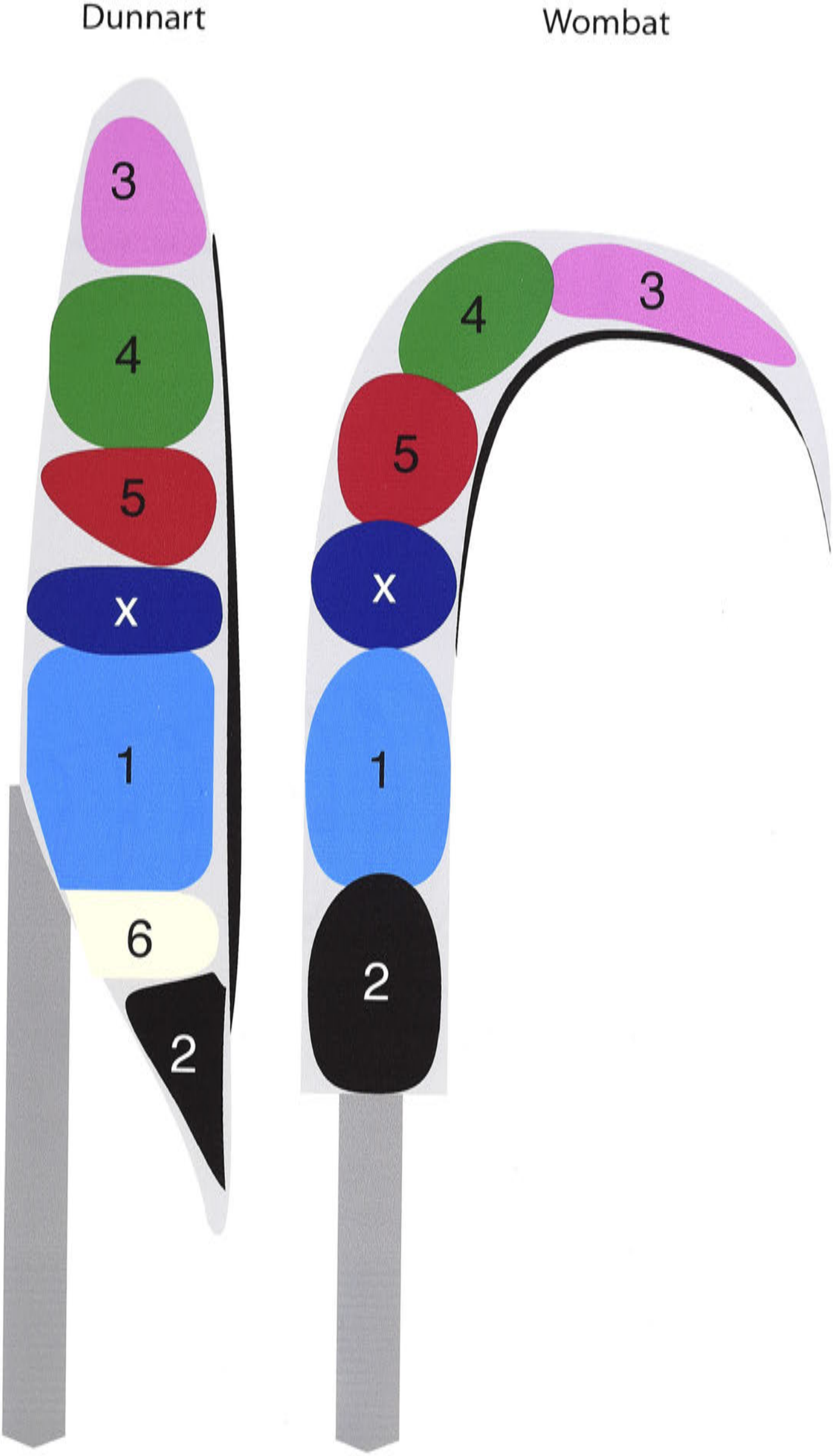
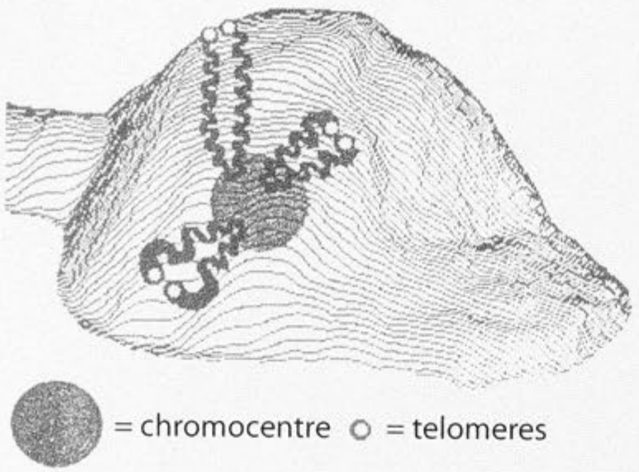
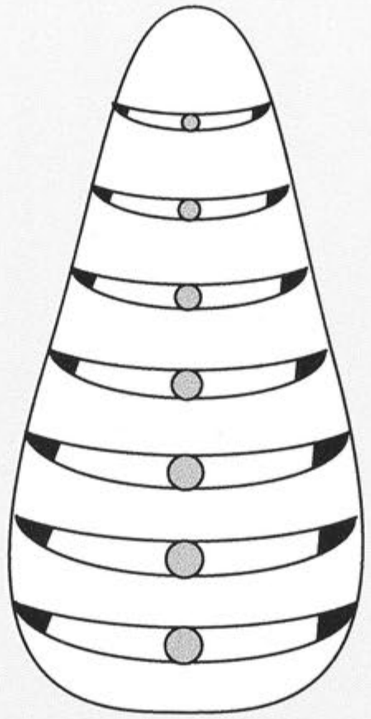


Figure 3.10: Chromosome positioning in marsupial sperm. The position of chromosomes 3, 4, 5, X, 1, and 2 are conserved in two marsupial species that diverged 50 million years ago .

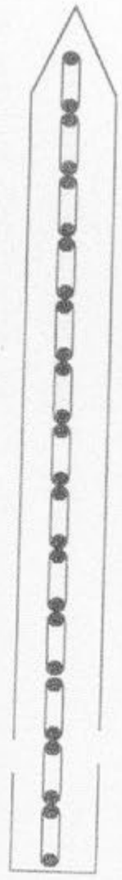


= chromocentre ○ = telomeres

EUTHERIANS
Humans
(Zalensky *et al,* 1995)



MARSUPIALS
M. eugenii and
S. crassicaudata



MONOTREMES
Echidna and Platypus
(Watson *et al,* 1996)

Figure 3.11: Hypothetical arrangement of telomeres and centromeres in mammalian sperm based on previous observations.

Primed in situ hybridization (PRINS) was also trialled for localization of the telomeric probe. It has the advantage of being a quick method, taking 6 hours, compared to the 1-2 day long FISH procedure. PRINS was carried out on Marmosops incanus, a south American marsupial with a 2n=14 karyotype and large interstitial telomeres (Svartman and Vianna-Morgante, 1998). PRINS revealed the large interstitial telomeres on all chromosomes (apart from chromosomes 6) that were reported by Svartman, 1998, but detected only a few telomeric signals at the ends of chromosomes (figure 3.12). The interstitial telomeric signals in the 2n=14 South American marsupial suggests that 2n=14 was not the South American ancestral karyotype. The method produced slides that were very dirty. I concluded that fluorescence in situ hybridization of telomeric probes was a more sensitive and useful method of observing telomeric sequences.

Hybridization of the telomeric probe to dunnart metaphase spreads showed telomeric signals on the ends of chromosomes as expected. There was also a strong interstitial signal at the centromeres of chromosomes 1, 2 and 6 suggesting that these chromosomes originated by centric fusions (figure 3.13). Such interstitial telomeric signals have previously been observed in rock wallabies (Metcalfe et al., 1997). The Y chromosome was also painted extremely brightly with the probe, indicating that a large number of telomeric repeats had accumulated on the Y chromosome, again a feature previously described for marsupial Y chromosomes (Metcalfe et al., 1998). No specific positioning of telomeres was observed in dunnart interphase cells or sperm (figure 3.13). The strongest telomeric signals were observed in the medial region and towards the periphery, but no consistent telomeric pattern was observed.

Telomeric probes were also hybridized to wombat and bandicoot metaphase spreads, and were localized only at the termini of all chromosomes (figure 3.13). In bandicoot interphase cells, telomeres were preferentially localized at the periphery of nuclei, suggesting attachment to the nuclear membrane (figure 3.13). Unfortunately hybridization of telomeric probes did not work in wombat sperm because preparations had been fixed in formalin. However, hybridizing telomeric probes to the morphologically similar sperm of koala (the closest relative to the wombat) showed no discernable pattern of telomeric positioning (figure 3.13). The telomeric probe would not hybridize to bandicoot sperm.

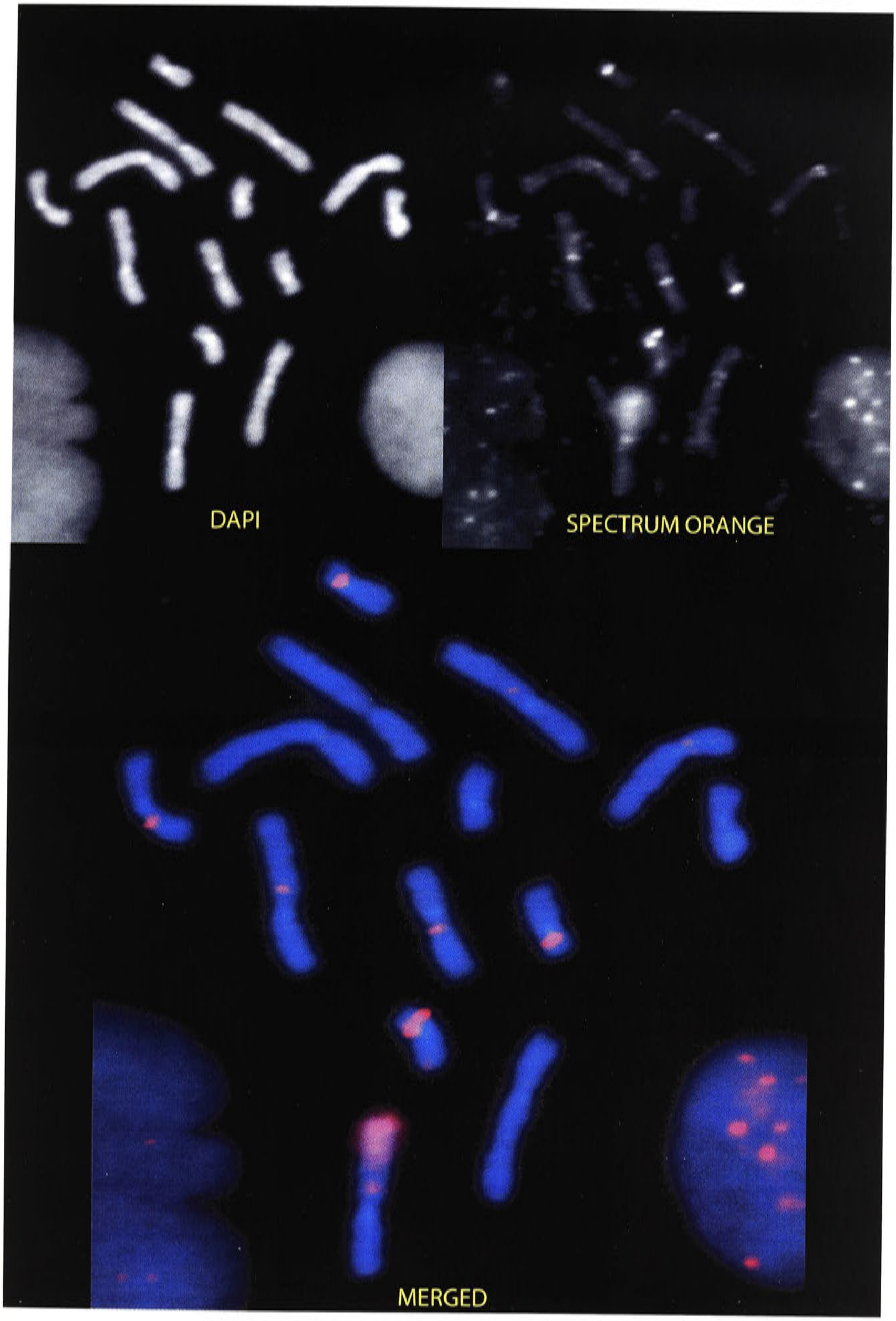


Figure 3.12: PRINS was used to localize telomeric sequences in *Marmosops incanus* (2n=14). Interstitial telomeric signals were observed on all chromosomes at varying intensities apart from the X. No terminal sequences were detected.

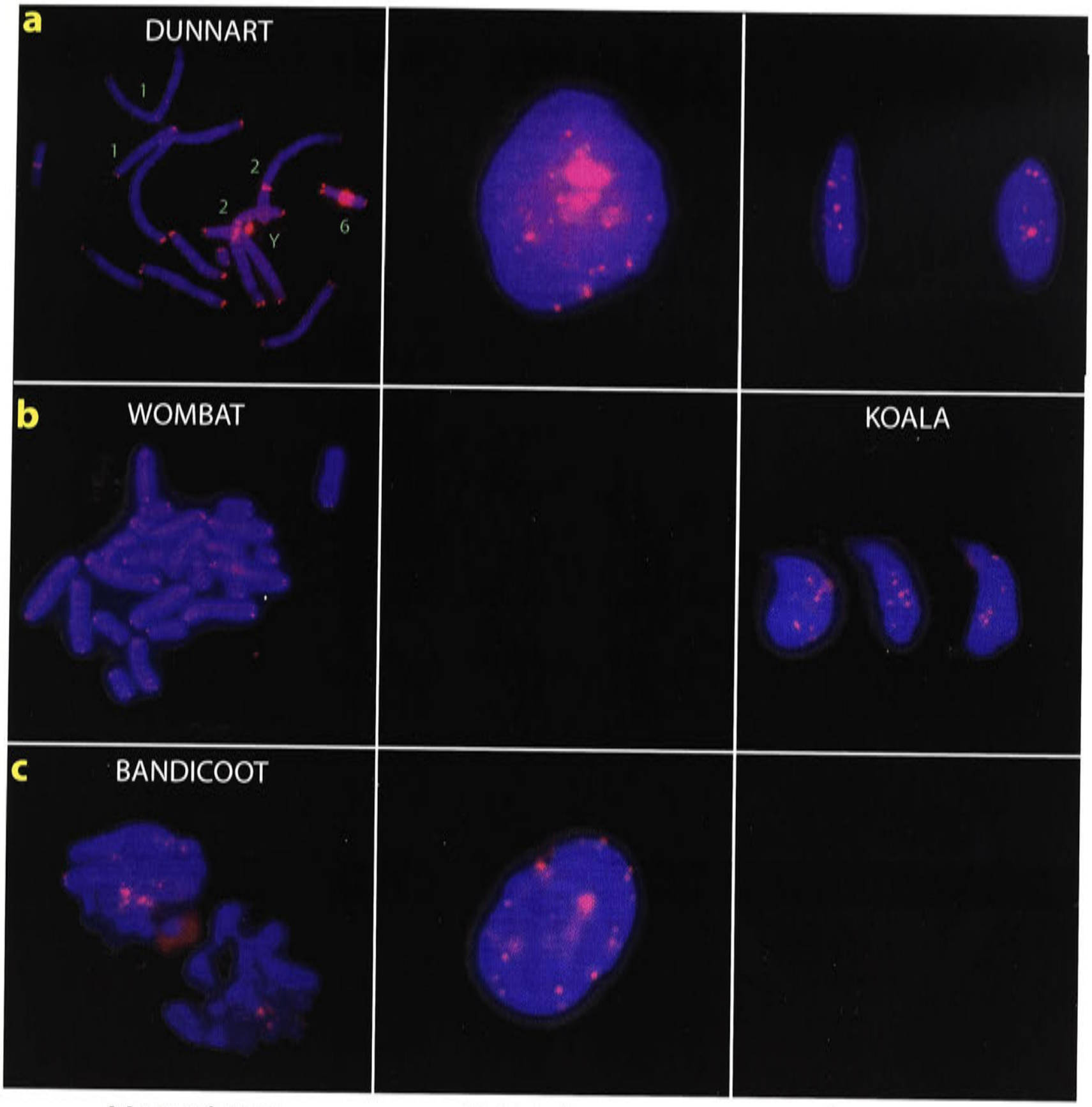


Figure 3.13: Telomere position in dunnart, wombat, koala and bandicoot metaphase, interphase and sperm. (a) In metaphase cells telomeric signals were observed at the end of each chromosome. Chromosomes 1, 2 and 6 also had interstitial telomeric sequences. The Y chromosome also had a large number of telomeric sequences. No consistent telomeric patterns were observed in interphase cells or sperm. (b) Wombat metaphase showed clear telomeric signals only at the ends of chromosomes. As telomeric signals could not be produced in wombat sperm, telomeric positioning was observed in sperm of the closely related koala. No consistent telomeric pattern was observed in koala sperm. (c) In a bandicoot metaphase, most signals were detected at the ends of a chromosomes. Telomeric signals in an interphase cell were predominantly at the periphery of the cell nucleus.

Telomeric probes hybridized to tammar wallaby metaphase spreads revealed bright signals at the centromeres, and small punctuate signals on the ends of chromosomes (figure 3.14). This was not unexpected, since other Macropus species have shown an accumulation of telomeric sequences at the centromere (Metcalfe et al., 1997). Chromosome 6 also had an interstitial telomeric signal on one of the chromosome arms.

Because the strong signals at centromeres overwhelmed the small terminal signals, it was not possible to study telomere position in tammar interphase cells or sperm. However, the hybridization of the probe to the centromeres allowed me to test my hypothesis that centromeres are lined up along the backbone to form a long chromocentre (figure 3.11). Hybridization of the telomeric probe to tammar sperm provided no consistent pattern. Highly variable signals were seen, with 3-5 signals in each sperm (figure 3.14). Although a few sperm had a single line of centromeric sequences running down the middle, most had two or three strong signals in the middle. This suggests that centromeres come together to form a chromocentre at the centre of the sperm nuclei.

3.2.2 Chromosome arrangement in platypus sperm

3.2.2.1 Platypus sperm morphology

The anterior and posterior ends of monotreme sperm is easy to distinguish. The anterior region of the platypus sperm forms a pointed cap (figure 3.2c). The tail extends from the posterior region that can be distinguished by its rounded end. The pointed end of the sperm is covered by the acrosome, which would point head on, when penetrating the egg for fertilization (figure 3.3c)

3.2.2.2 Platypus chromosome- specific sorting and paints

The karyotype (2n=52) of the platypus consists of 6 large autosomes, the sex chromosomes, many small chromosomes and some unpaired elements. The homologous partners of the unpaired elements have undergone translocations to other chromosomes. The X chromosome in male platypus undergoes homologous pairing with element E2, forming the first 2 elements of the meiotic chain in meiosis.

3.2.2.3 Chromosome arrangement in Platypus sperm

Paints were prepared for two chromosomes (the X and a small autosome) that were successfully flow-sorted from platypus, and used to probe the chromosome

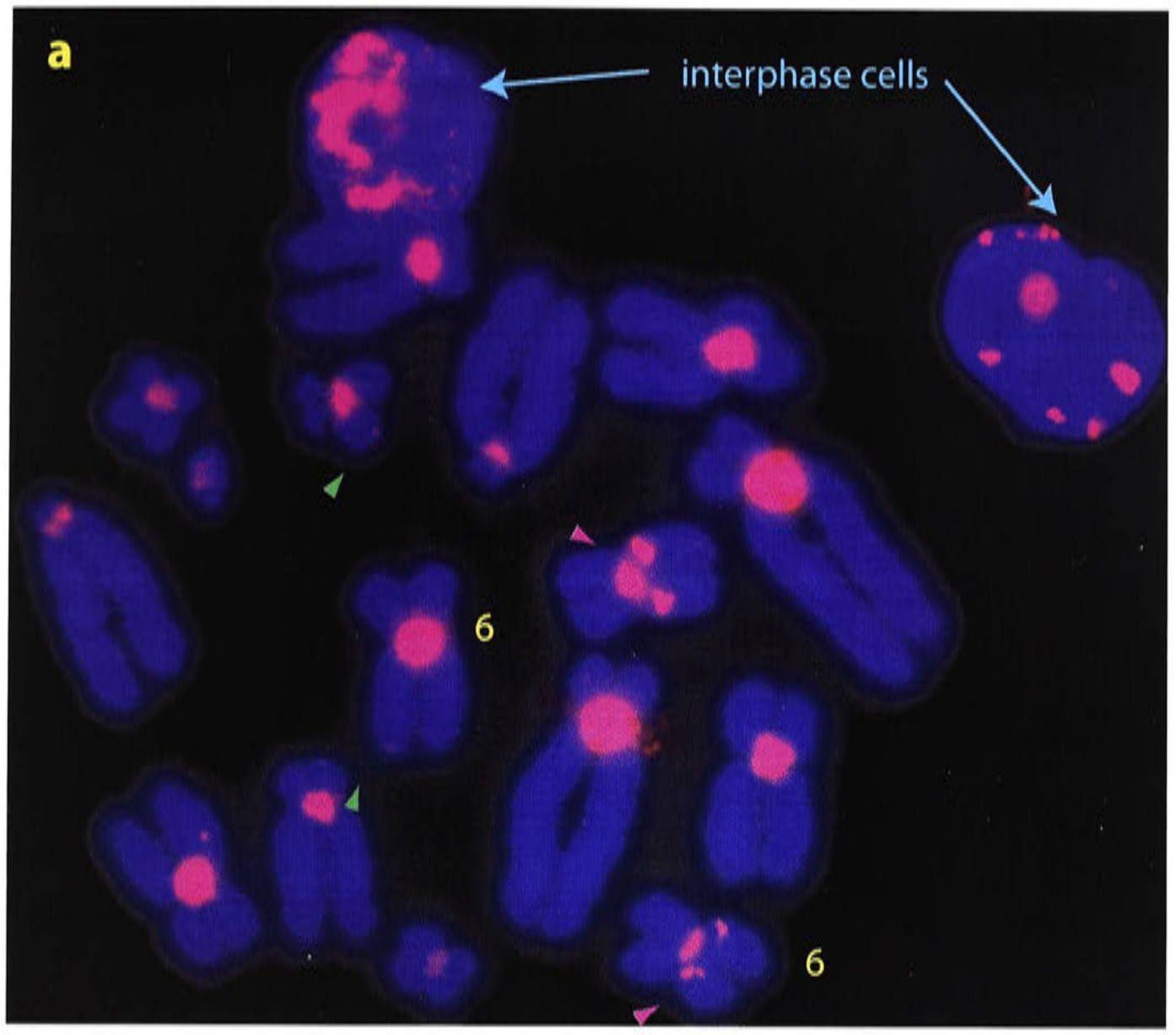


Figure 3.14: Telomere probes in the tammar wallaby. (a) Metaphase cell showing strong signals at centromeres of all chromosomes, but few telomeric signals at the ends of chromosomes, as these small telomeric signals were overwhelmed by the centromere signals (►).There were also interstitial signals on chromosome 6 (►). (b) Tammar sperm showing many different patterns of centromeric positioning.

positions in at least 30 platypus sperm.

The flow-sorted X chromosome paint was hybridized to male metaphase spreads (Fig 3.15a). It hybridized to the X, and also a small unpaired telocentric element. This represents the element E2 seen to pair with the short arm of the X at meiosis (Grutzner, Rens unpublished). A second paint made from the flow-sorted unpaired element E2 hybridized to Xp as well as to E2, which is a putative Y chromosome.

Hybridization with the probe produced a signal in 80% of sperm; failure to detect signal was attributed to hybridization and incorporation problems. As expected, only one signal was seen in each sperm, showing that the X segregates from E2. The X chromosome was observed to be preferentially at the anterior of the spermhead in 75-80% of platypus sperm (table 3.4 and figure 3.16). This finding is consistent with the findings from *in situ* hybridization of Watson *et al* (1996) that the X chromosome had a non-random position at the anterior region of the spermhead (figure 3.17). It was not possible to distinguish the X and E2 signal, but the uniformity of signal position implied that the X chromosome and E2 were located in the same region of sperm.

The X paint (hybridizing to the whole of the X chromosome) and E2 hybridized to 80% of sperm. Despite the difference in size, it was impossible to determine, from the size of the signal, whether the sperm contained the X chromosome or the E2 element because 2-dimensional imaging of the sperm do not give an accurate representation of chromosome size, also because of differences in intensities of the signal and background.

A paint derived from a flow-sorted small autosome (pair 17-21) was also painted to platypus sperm. The signal was found at the posterior region of the spermhead in more than 80% of sperm (figures 3.16 and 3.17). Variation in signal position may be due to mis- interpretation of sperm orientation.

3.2.2.4 Telomeres in platypus sperm

Telomeric oligonucleotides were hybridized to platypus somatic cells and sperm. Platypus metaphases showed small punctuate telomeric signals at the ends of most chromosomes (figure 3.18). Chromosome 1 also had a bright interstitial telomere signal on 1q, suggesting an ancient telomeric fusion. No pattern of telomeric distribution was observed in platypus interphase cells.

Telomeres were not clustered in sperm. There was an even distribution of telomeric sequences along the length of the platypus spermhead. Between 20 and 30

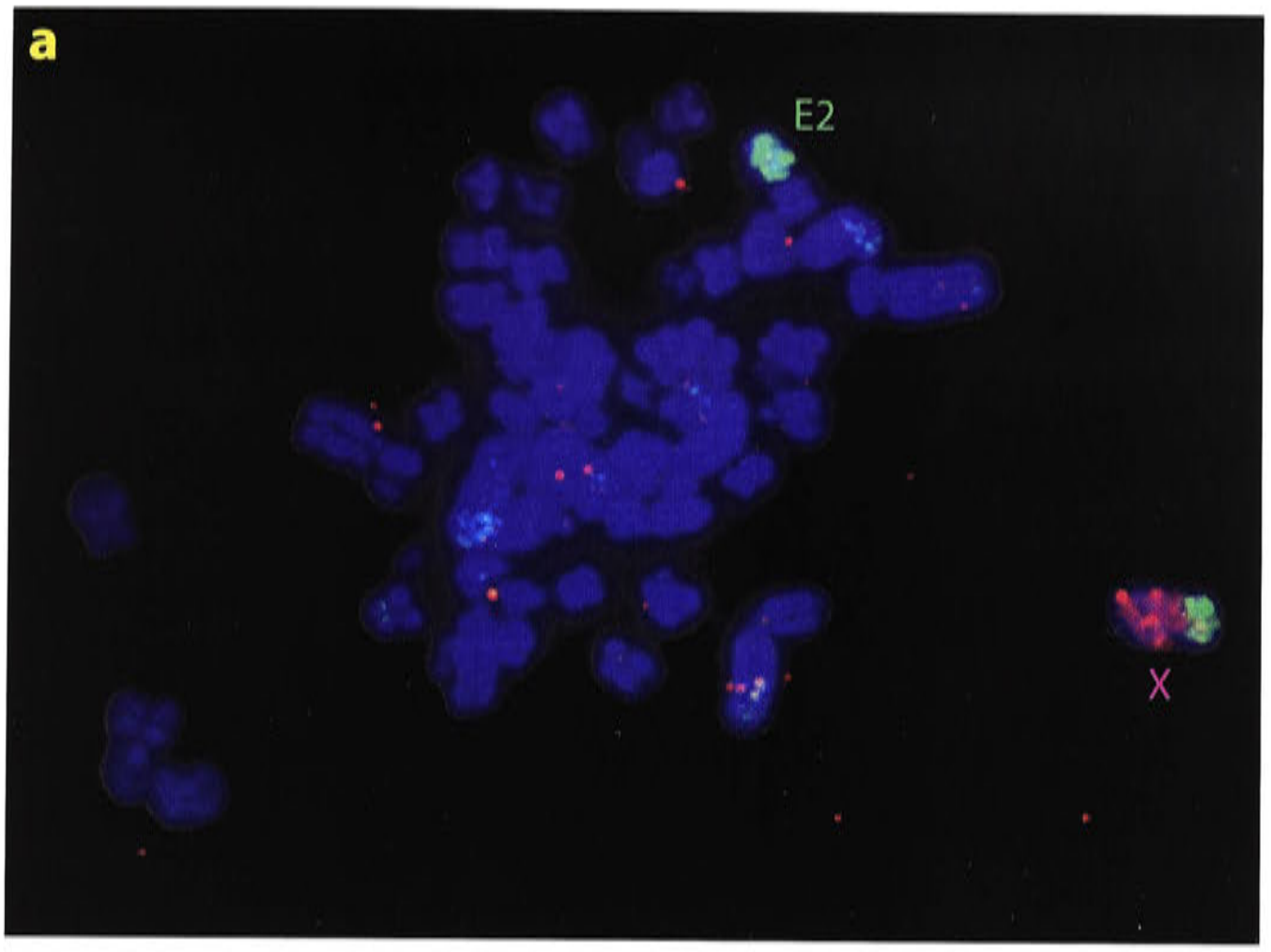


Figure 3.15: Paints used to observe chromosome position in platypus sperm. (a) Double painting with two X chromosome paints were used, The E2 paint (green) hybridized to E2 and to the X. The whole X paint (red) hybridized to the whole X chromosome and to E2. (b) A small autosome paint one of pair 17-21 hybridized to a pair of small autosomes. (a is the merged picture from figure 4.5)

Table 3.4: Position of signal in platypus sperm.

chromosome	Numbers (%) of sperm with signal			
	anterior	medial	posterior	Total
X	**30 (73%)**	9 (22%)	2 (5%)	41
17-21	0 (0%)	4 (13%)	**28 (87%)**	32

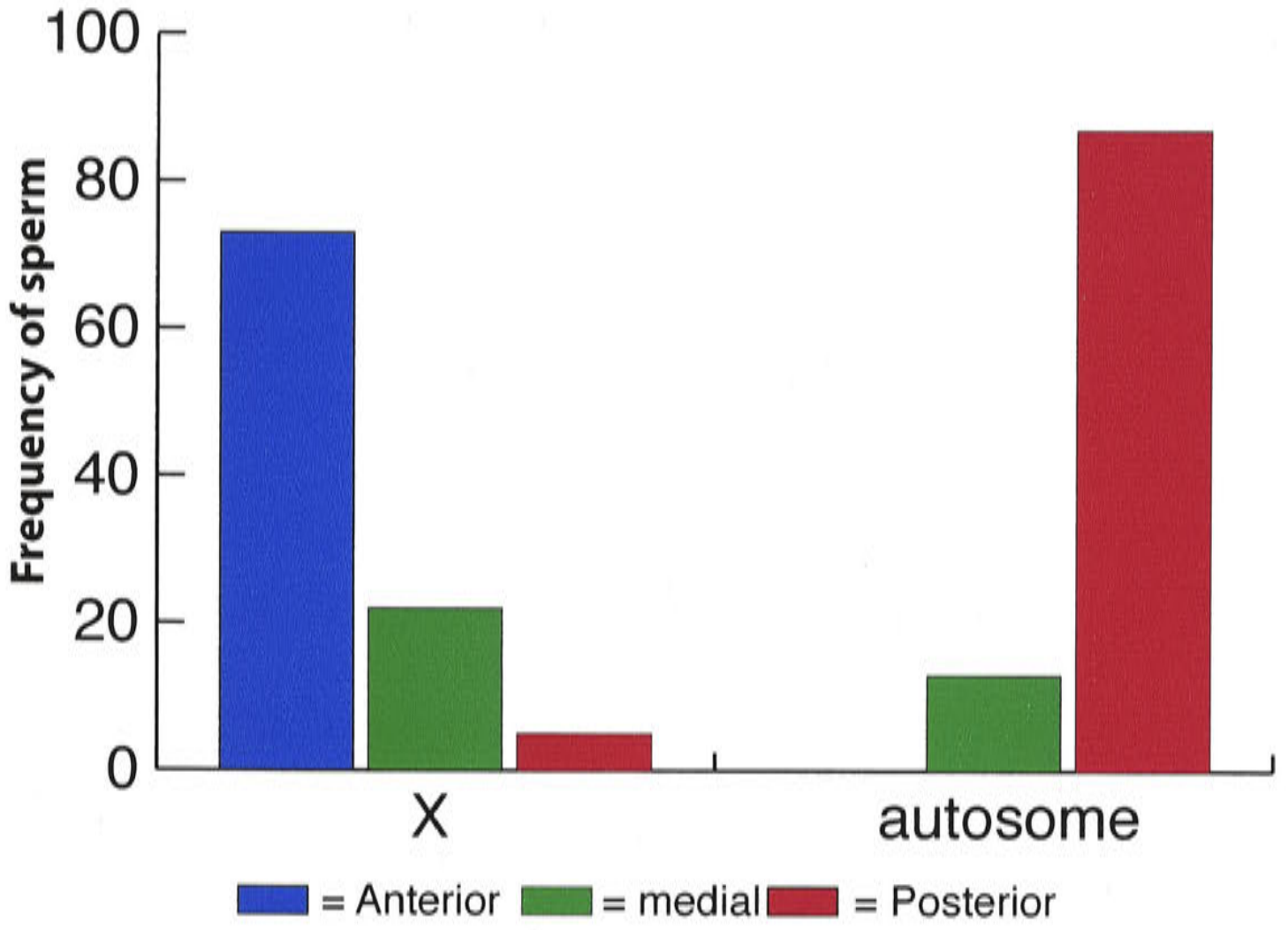


Figure 3.16: Frequency of sperm with signal in different regions. For the X chromosome 41 signals were seen in 47 sperm. For the paired 17-21 autosome 32 signals were seen in 34 sperm.

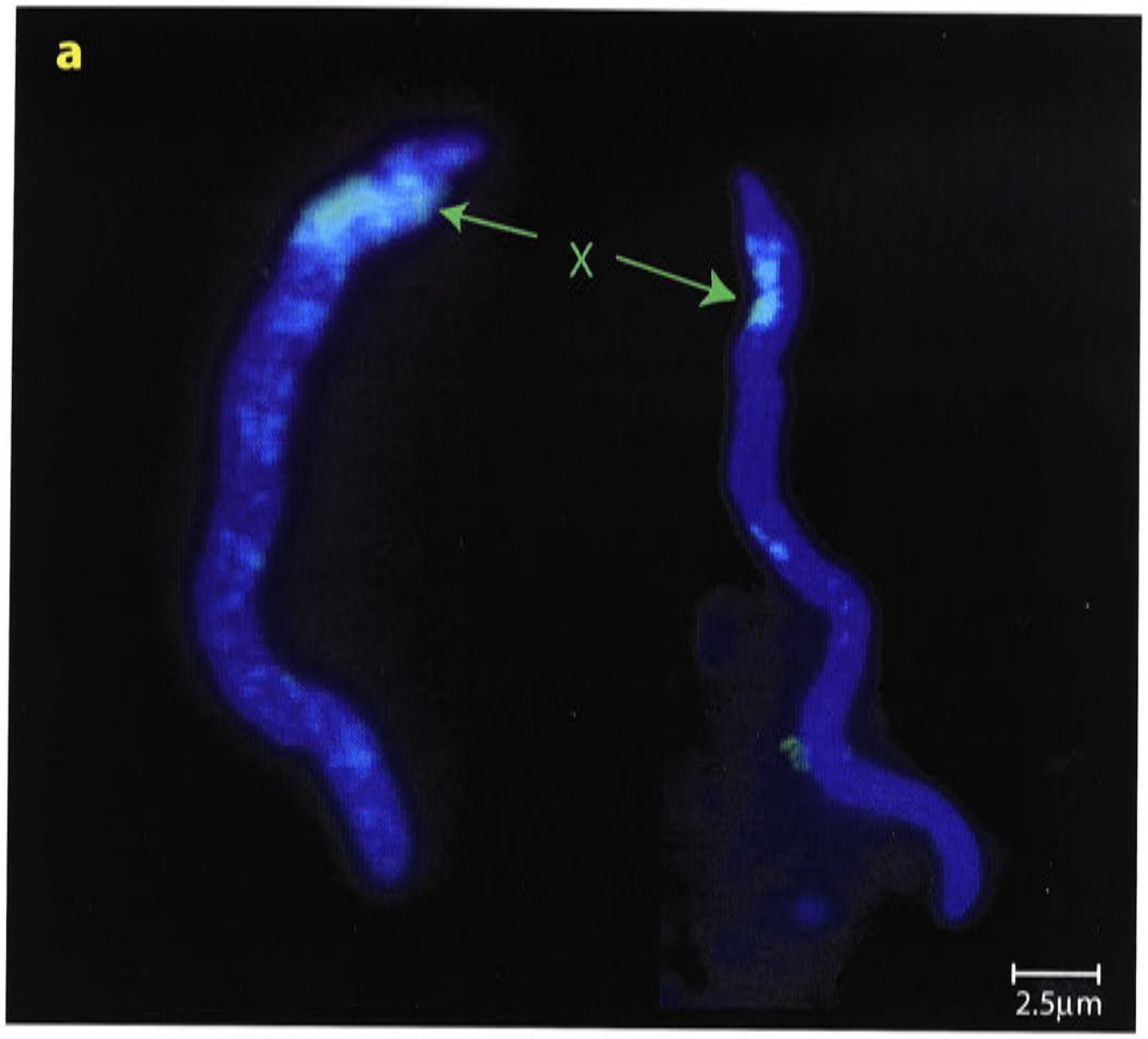
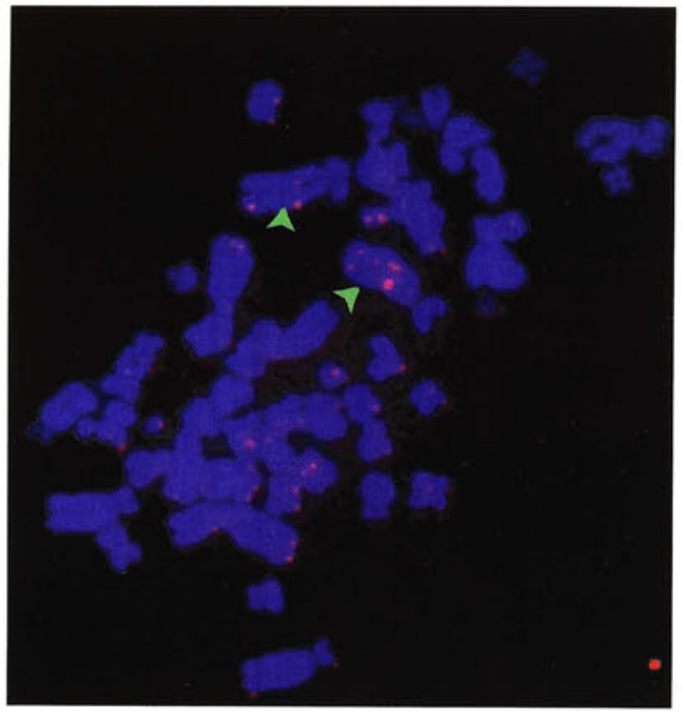
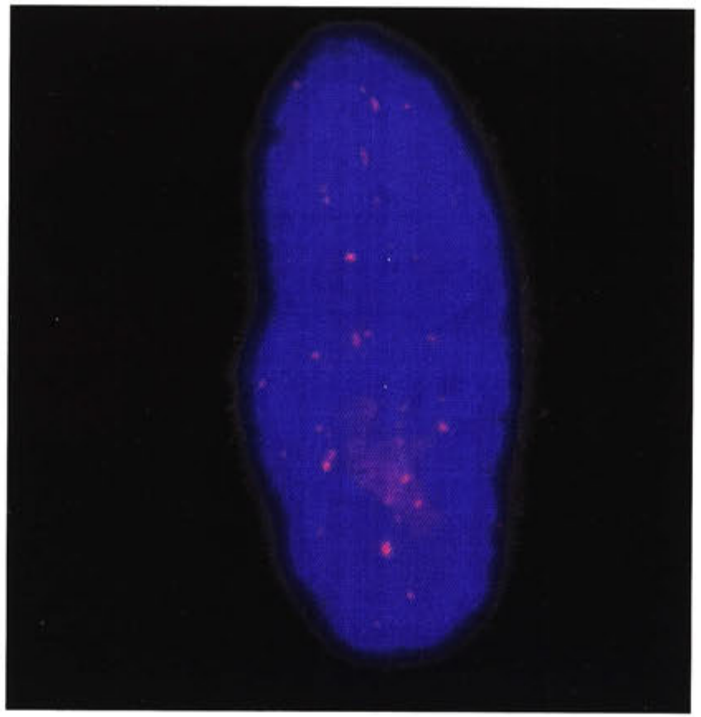


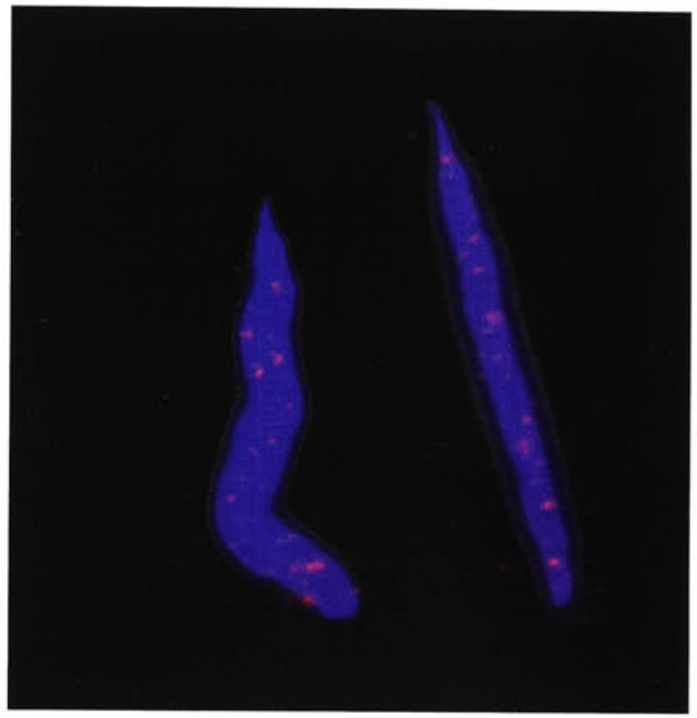
Figure 3.17: Chromosome position in platypus sperm orientated with anterior end up. (a) The X chromosome was preferentially located at the anterior of the nucleus. (b) The autosomal pair 17-21 lies preferentially at the posterior of the nucleus.



Metaphase



Interphase



Sperm

Figure 3.18: Telomeric signals in platypus somatic cells and sperm. Most signals were found at the ends of chromosomes. Chromosome1q has an interstitial telomeric siqnal (➤). Telomeric telomeres were distributed evenly at interphase and in platypus sperm.

signals were counted in a spermhead. This corresponds with the observations of Watson *et al* (96). Given that the haploid sperm should have 26 chromosomes (therefore 52 telomeres), this suggests that that telomeres from one chromosome lie juxtaposed with the telomeres from another chromosome, suggesting that the chromosomes lie end-to-end along the sperm (figure 3.11 and 3.18).

3.2.3 Chromosome arrangement in chicken sperm

3.2.3.1 Chicken sperm morphology

Chicken sperm are long and fibrillar, with the acrosome at one end of the sperm. The anterior and posterior of the spermhead can be distinguished by light microscopy or phase contrast (figure 3.2d). Light microscopy allows the tail to be seen, whereas phase contrast allows the acrosome to be distinguished. Fertilization requires the anterior of the spermhead making contact with the egg (figure 3.3d).

3.2.3.2 Chicken chromosome- specific sorting and paints

The chicken karyotype (2n=78) consists of nine macrochromosomes and many microchromosomes. The sex chromosomes of the chicken differ from mammals. Females are heterogametic (ZW) and males homogametic (ZZ).

Four flow-sorted chicken chromosomes were used to observe chromosome position in chicken sperm. Chromosomes 2, 8 and 9 were chosen to represent a large chromosome and two small chromosomes, in order to observe if chromosome size affected chromosome position within the cell. The Z chromosome allowed a comparison of sex chromosome position between chickens and mammals.

3.2.3.3 Chromosome arrangement in chicken sperm

Painting the four flow-sorted chicken chromosomes (2, 8, 9 and the Z) to chicken sperm (194 in total) produced a result very different from that seen in marsupials and monotremes. Since the anterior and posterior regions could not be identified, the position of the signals relative to the middle was scored to determine chromosome position in a spermhead. This was done by dividing the sperm into six sections *a*, *b*, *c*, *c*, *b* and *a*, with *a* representing both ends of the sperm, *b* representing the medial-anterior region and the medial-posterior region, and *c* representing the medial regions (figure 3.19). Comparing frequency of sperm with signals in *a*, *b*, and *c* showed that no chromosome was found in a particular region more than 45% of the time (figure 3.19).

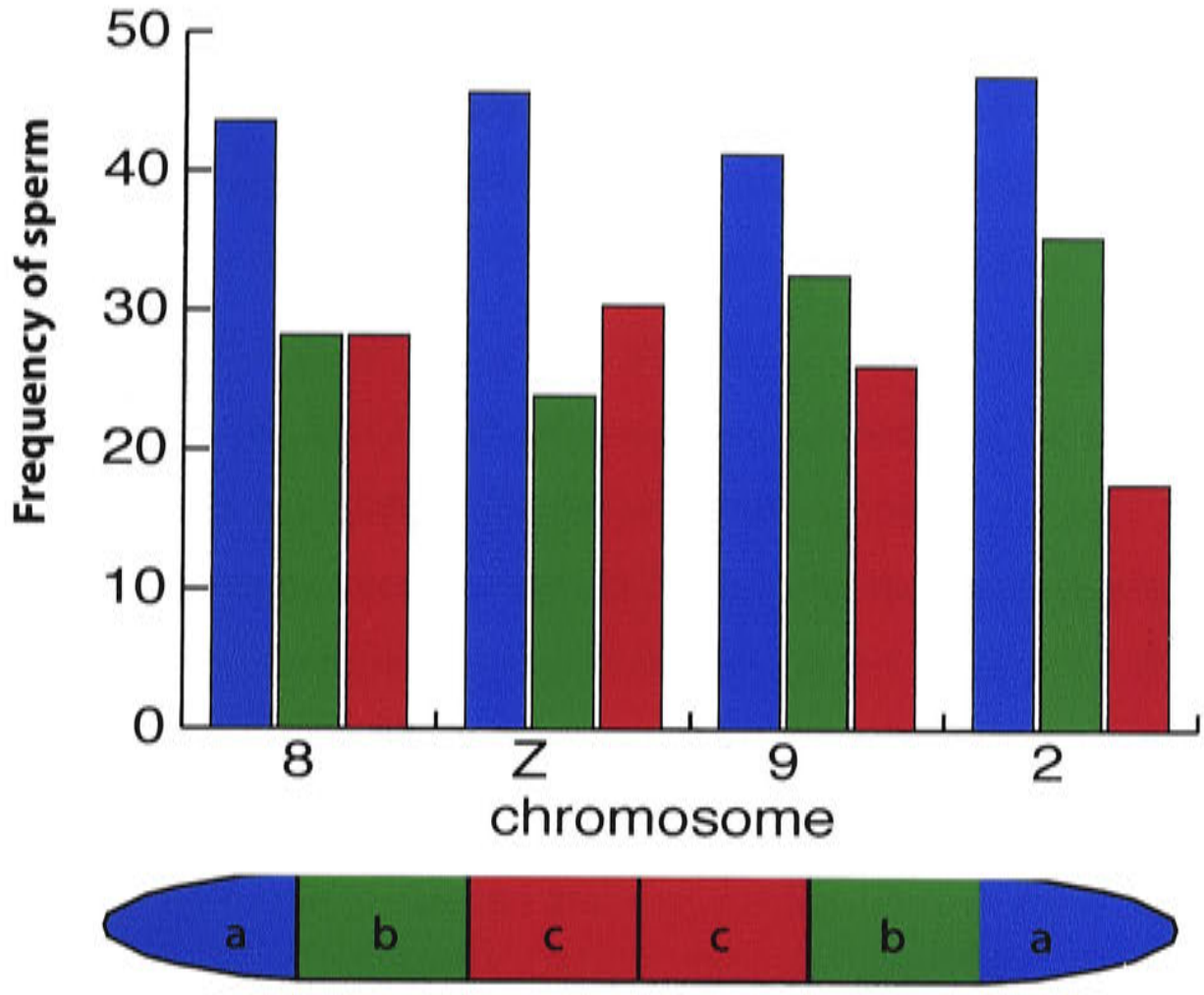


Figure 3.19: Chromosome position in chicken sperm. The graph shows no strong localization of a chromosome to a particular position, although there was a slight tendency for the chromosomes to be positioned at the ends of the sperm.

Table 3.5: Relative positions of chromosomes in chicken sperm.

Ch	Frequency (%) of sperm with signal					
	different ends	end/ middle	middle/ end	both in middle	Same end	Total
9/2	5 (22%)	5 (22%)	8 (34%)	3 (13%)	2 (9%)	23
9/8	2 (13%)	1 (6%)	5 (31%)	5 (31%)	3 (19%)	16
8/Z	1 (9%)	3 (27%)	4 (37%)	2 (18%)	1 (9%)	11
9/Z	7 (24%)	3 (10%)	8 (28%)	6 (21%)	5 (17%)	29

Multicolour painting with two chromosome-specific probes clearly showed that their relative positions were inconsistent in different sperm (figure 3.20 and table 3.5). For example, in some sperm chromosome Z and 9 were observed together in the medial region, whereas in other sperm they were at opposing ends (figure 3.20). Although double painting provided clear evidence of inconsistent chromosome positioning, there was a slight tendency for all four chromosomes to be positioned at the ends of the sperm (figures 3.19). A possible explanation for this is discussed below.

In contrast to the conserved position of the sex chromosomes in mammals, the Z chromosome was not preferentially located at the anterior region of the spermhead (figure 3.20). The Z chromosome was observed at the ends of some sperm and the medial region of other sperm.

Thus in contrast to the conserved and non-random chromosome arrangement in mammals, the chromosome arrangement in chicken sperm showed no consistent chromosome position.

3.2.3.4 Telomeres in chicken sperm

When chicken fibroblast metaphases were hybridized with the telomeric probe, most chromosomes showed small punctuate telomeric signals at the ends of chromosomes. Particularly striking was the intensity of signals on the microchromosomes (figure 3.21).

Thus telomere-specific probes were ideal to study microchromosome position in chicken sperm. The telomeric probe hybridized to chicken sperm produced an uneven distribution of signals in one large and one or two smaller clumps (figure 3.21). The bright clusters in sperm suggest that microchromosomes are clustered together within the spermhead. The large cluster of signal within the medial region (regions *b* or *c*) never localized to the ends of the spermhead (regions *a*). This explains why the macrochromosomes were preferentially at the ends.

Thus macrochromosomes have no specific pattern of distribution within chicken sperm, apart from a preference for the ends, and the microchromosomes tend to lie in the medial regions of the spermhead.

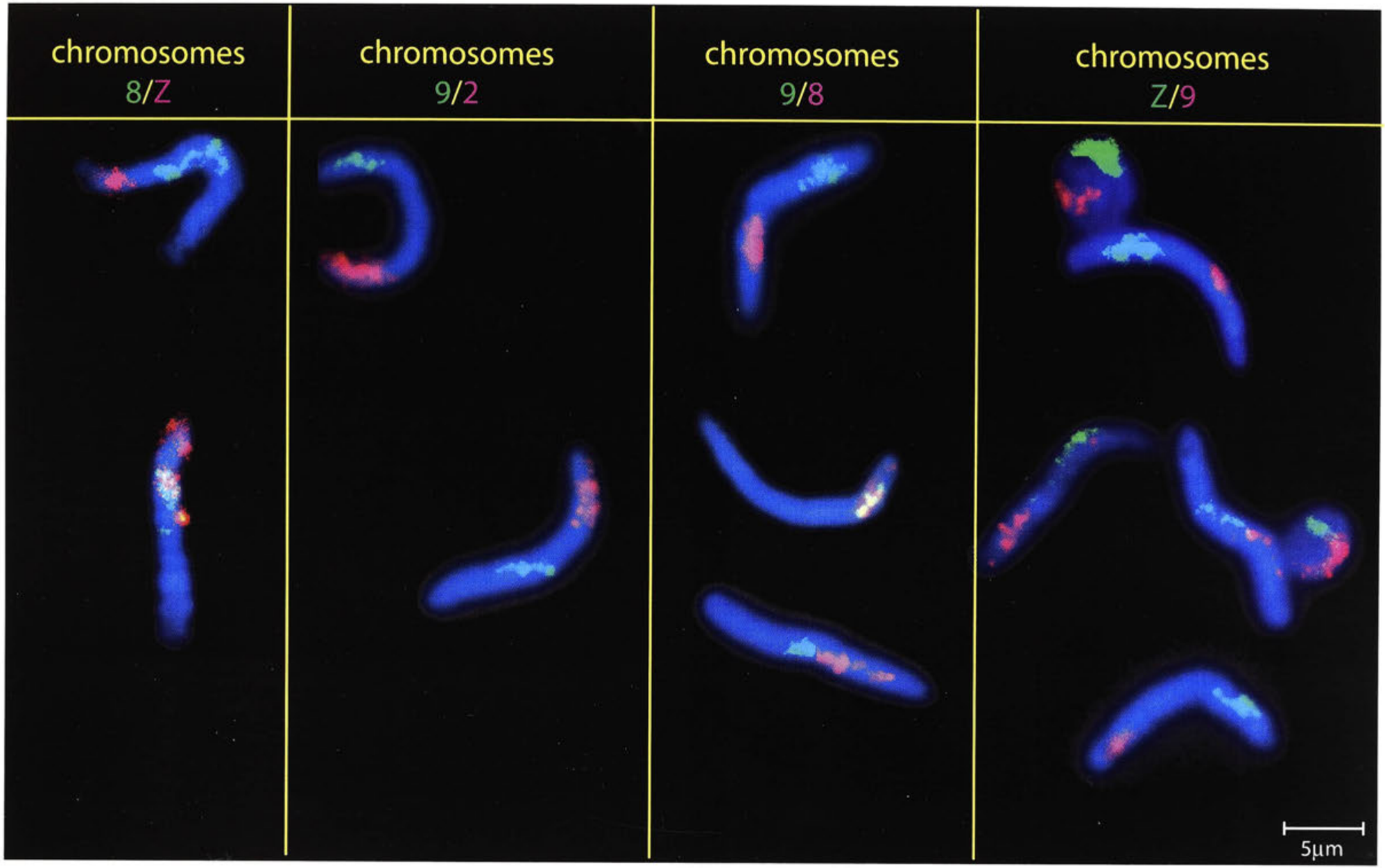
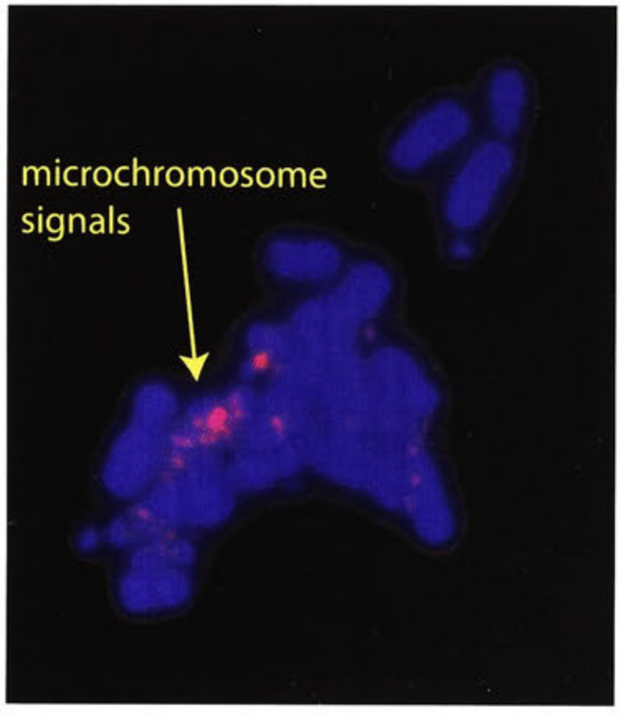
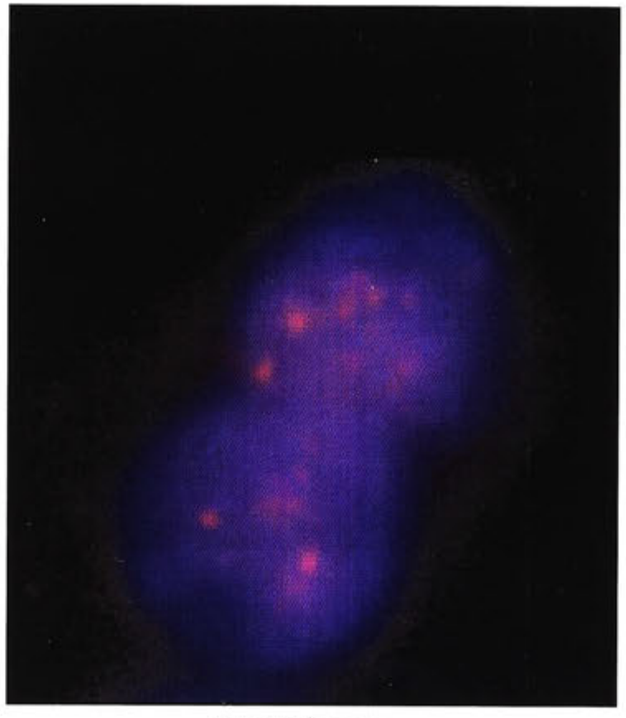


Figure 3.20: Double painting of different chromosome-specific probes in chicken sperm. Relative positions are inconsistent between sperm. The coloured signal in the sperm corresponds to the same coloured chromosome number.



Metaphase



Interphase

Sperm

Figure 3.21: Telomeric signals in chicken somatic cells and sperm. Microchromosomes contain high amounts of telomeric sequences, so the telomeric probe was used to study microchromosome position within chicken interphase cells and sperm. Microchromosomes were inconsistently positioned in mitotic metaphases and interphase cells and were preferentially positioned in the medial region of the chicken sperm.

3.3 Discussion

In this study I addressed the organization of chromosomes in marsupial, monotreme and chicken sperm. It is now evident that a non-random chromosome arrangement is found in marsupial sperm and this arrangement is conserved between two marsupials that diverged 50-60 million years ago. This suggests an important function for the order observed to be conserved.

The position of the X chromosome between eutherians, marsupials and monotremes also seems to be conserved in relation to sperm-egg contact. No distinct arrangement of chromosomes was observed in chicken sperm, suggesting that the non-random arrangement of chromosomes observed in mammalian sperm, may have a mammalian specific function.

3.3.1 Non-random chromosome arrangement in mammalian sperm

This study observed the chromosome arrangement in wombat and monotreme sperm to test the generality of a non-random chromosome arrangement in sperm across all three mammalian groups. The results demonstrated clearly that chromosome arrangement is non-random in wombat and monotreme sperm. Each chromosome in the wombat had a consistent chromosome position; for example, chromosome 3 was preferentially positioned at the anterior of the spermhead, whereas chromosome 2 was preferentially positioned at the posterior. In the fibrillar monotreme sperm, the X chromosome and a small autosome also had non-random positions. The X chromosome was preferentially found at the anterior of the spermhead, whereas the autosome was preferentially positioned at the posterior.

Therefore I conclude that chromosome arrangement is non-random in all three groups of mammals. It must therefore have evolved before the divergence of the three mammalian groups 170 million years ago. The conserved non-random arrangement may represent a conserved mammal-specific function.

In this study, a model for marsupial chromosome organization in sperm was proposed, with telomeres at the periphery of the sperm nucleus, centromeres running along the centre of the sperm nucleus and chromosomes forming a U shape within the sperm nucleus. However, hybridization of a telomeric probe provided no evidence for any such arrangement. Telomeric signals were dispersed through the spermhead.

Signals were found at the periphery but also within the centre of the nucleus; however focusing in and out of the focal plane often suggested peripheral position of the telomeres. 2-dimensional analysis limited the ability to properly analyze telomeric positioning within the sperm. Obviously 3-dimensional analysis is essential for the accurate determination of telomere position in marsupial sperm.

In the proposed model, centromeres were aligned along the centre of the nucleus. However, centromeric positioning in tammar wallaby sperm did not indicate this and hybridization with a probe that detected centromeres on tammar chromosomes showed that centromeric position was highly variable, though in most case two to three signals were observed, with one large signal towards the centre of the sperm nucleus. This arrangement is similar to that observed in human sperm, where centromeres come together at the centre of the nucleus to form a chromocentre (Zalensky et al., 1995).

Although it was hard to differentiate the different wombat sperm morphologies after DAPI staining (figures 3.5, 3.7), there was no difference in chromosome position obvious between the different variations. Therefore if one sperm type has a higher probability of reproductive success, it is not due to differences in nuclear organization. It is more likely that other features affect reproductive success; for example, different levels of sperm motility, or abnormal acrosomes.

3.3.2 Chicken sperm maturation and nuclear organization

In chicken sperm, chromosomes occupied inconsistent positions, although macrochromosomes tended to be positioned at the ends of the sperm and microchromosomes clustered in the middle (regions *b* and *c*). This arrangement of microchromosomes in the medial region of the spermhead and macrochromosomes towards the ends reflects their arrangement in interphase cells (Habermann et al., 2001), in which microchromosomes are clustered at the interior of the nucleus, and macrochromosomes are randomly positioned around the periphery. The central position of the microchromosomes could result from the initiation of sperm elongation at random positions on the membrane. This would allow peripheral chromosomes in one region to enter the spermhead first, followed by the microchromosomes in the interior and then by macrochromosomes positioned furthest away from the point at which elongation began (figure 3.22).

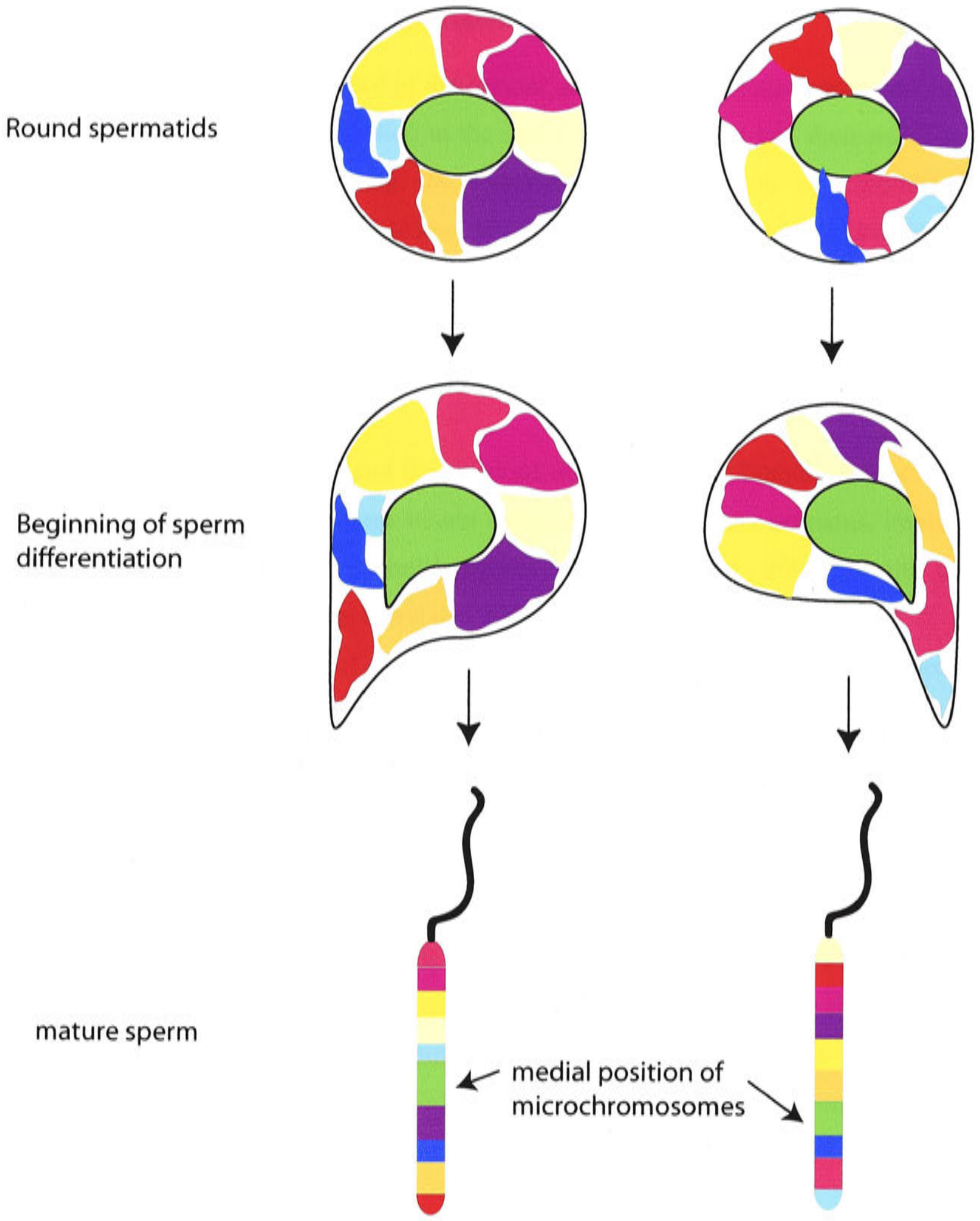


Figure 3.22: Hypothesis for the development of chicken sperm nuclear organization. As in interphase cells, microchromosomes (green) are preferentially positioned in the interior of the round spermatid, whereas macrochromosomes (other colours) are randomly positioned around the periphery of the nucleus. Elongation of the spermatid is initiated at a random position on the membrane and will determine the order in which macrochromosomes enter the tube. As microchromosomes are preferentially positioned in the interior of the round spermatid, they will have a tendency to be positioned in the medial region of the sperm head.

3.3.3 Conservation of nuclear organization in sperm

To explore the significance of the regular chromosome arrangement in mammal sperm, I investigated its conservation in the sperm of the wombat and dunnart. These species were ideal for assessing the evolutionary conservation of chromosome arrangement in sperm nuclei because they have virtually identical karyotypes. Chromosome position was found to be conserved between the two species, suggesting an important function for the arrangement. The chromosome arrangement from the anterior to the posterior of the sperm was chromosomes 3 – 4 – 5 – X – 1 and 2 in both the dunnart and the wombat.

These results greatly extend the previous observation that human chromosomes 18 and 19 have conserved positions within interphase cells of other primates, implying that the arrangement is at least 30 million years old (Tanabe et al., 2002). In this case the position of the chromosomes correlates with chromosome activity.

Therefore the position of a chromosome in sperm may influence its position in the zygote, which in turn influences its future activity. For example, chromosome 3 may be positioned at the anterior of the spermhead to set up its chromosome domain within the zygote. As the paternal nuclei enter the egg, chromosome positions may already be set for the zygote. Chromosome 3 found at the anterior, and chromosomes 1 and 2 found at the posterior may take up a peripheral position within the paternal pronucleus, whereas the cluster of chromosomes in the medial region (4, 5, and X/Y) take up an internal position within the paternal pronuclei. However, it could also be the other way around. This chromosomal arrangement in the zygote may directly affect gene expression, with the internal position of 4, 5, and the X or Y chromosome indicating chromosomal activity, whereas chromosomes 3, 1 and 2 would be destined for inactivity.

Slight variation was observed in chromosome positions for both species. This can probably be attributed to the limitation of observations in 2-dimensions. Although the shape of the sperm gave useful reference points for determining chromosomal position, it was not always straightforward to determine the orientation of the spermhead. For example, a sperm fixed at a 45 degree angle from the surface of the slide could produce an inaccurate interpretation of the chromosome position.

The sperm morphology is very different between dunnart and wombat. The

conservation of chromosome arrangement in the arrow shaped spermhead of the dunnart and the hook-shaped spermhead of the wombat implies that the nuclear organization is not influenced by sperm morphology. The same conclusion is reached for sperm of chicken and platypus, which have a very similar morphology, but very different nuclear organizations. Monotremes have highly ordered sperm nuclei with a tandem arrangement of chromosomes that take up specific positions within the spermhead. In contrast, chicken does not show the same level of organization, apart from the tendency for macrochromosomes to be positioned at the ends and microchromosomes towards the medial region of the spermheads.

3.3.4 Conservation of the sex chromosome position in mammalian sperm

I observed that in platypus the X chromosome paint produced a signal at the apex of the fibrillar platypus sperm, confirming the indirect observations of Watson *et al* (96). Since this paint also detects the pairing partner of the X (element E2 of the meiotic chain, and the putative Y), this localization reflects both. It was not possible to distinguish the X and E2 signal by size, but the observation of 80% of sperm with apical label must mean that the X and E2 segregate into different sperm.

In wombat and dunnart sperm, the X and Y occupy a medial position, which, after a morphological transition to a T-shape during maturation, becomes the point of first contact with the egg on fertilization (Breed, 1994). In humans too, the X chromosome has an anterior position in the sperm nucleus (Hazzouri et al., 2000b) (figure 3.23). Thus the position of the X chromosome in relation to sperm-egg contact has been conserved over 170 million years of mammalian evolution. More interesting still is the inconsistent position of the chicken Z chromosome, suggesting a mammal specific function for the apical position of the mammalian X chromosome.

What might this function be? One possibility is that position may be important for X chromosome inactivation, perhaps as part of a recognition mechanism for paternal X-inactivation. Paternal X inactivation occurs in marsupials, as well as in extraembryonic tissue of mice and rats, and is thought to be ancestral. Thus, in the mammalian ancestor, the position of the X chromosome may have played an important role in paternal X-inactivation. During evolution eutherians developed a different process for inactivation in the embryo, but retained paternal inactivation in the earliest differentiating tissues (Monk and Harper, 1979).

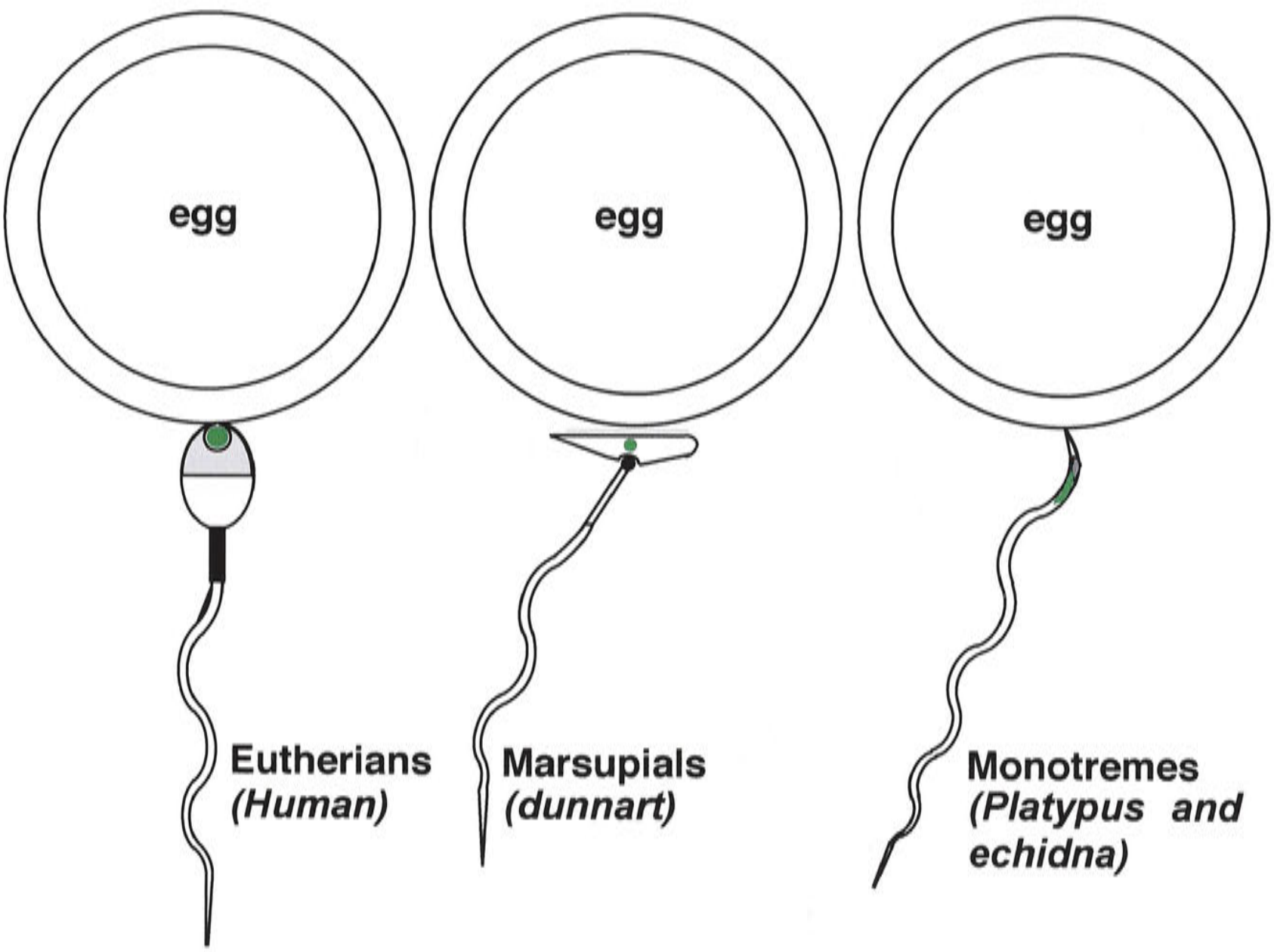


Figure 3.23: Mammalian sperm-egg interactions and the position of the X chromosome. In all three mammalian groups the X (green) is positioned to be the first part of the paternal genome to enter the egg. The acrosome is shaded in grey.

What makes this idea even more persuasive is that the Z chromosome in birds does not undergo inactivation, and has no consistent position in sperm, so does not enter the egg first.

3.4 Summary

This study had four specific aims, one, to determine whether chromosome arrangement was non-random in marsupials and monotremes, two, to determine whether this was a mammal-specific characteristic, three, to test whether the non-random arrangement was conserved in related mammals, and four, to observe whether the X chromosome position was conserved in mammals.

I discovered that chromosomes are arranged in sperm of marsupials and monotremes implying that a non-random arrangement of chromosomes is a feature of mammalian sperm nuclear organization. However, chromosomes are arranged in no consistent order in chicken sperm, although microchromosomes tend to lie in the medial region. This suggested a new model of how the nuclear organization in chicken sperm may be produced. My observation that a non-random arrangement is conserved in mammals, but not birds suggests an important mammal-specific function for the non-random arrangement of chromosomes observed in mammalian sperm. Furthermore, comparison of the chromosome arrangement of dunnart and wombat showed a conserved arrangement of chromosomes, implying an important function for the arrangement. One possibility is the arrangement in sperm sets up important chromosome and functional domains within the zygote.

In all three mammalian groups the X chromosome is found at the point of contact between the sperm and the egg. Therefore the X chromosome is the first part of the paternal genome to enter the egg. This may allow a specific imprint or position to determine inactivation of the paternal X chromosome, as observed in marsupials.

Future work in this field should require the use of a laser scanning confocal microscope and 3-D imaging software allowing an accurate reconstruction of chromosome position in sperm. Such reconstructions would allow a better overall understanding of chromosome organization within the sperm head.

CHAPTER 4: CONSERVATION OF THE MAMMALIAN X CHROMOSOME.

4.1 Introduction

In chapter 3, I reported that the X chromosome had a conserved position in sperm of marsupials and monotremes at the point of contact between the sperm and the egg. The X chromosome is therefore the first part of the paternal genome to enter the egg. Thus the position of the X chromosome in sperm may help to set up paternal X-inactivation.

For the conserved position of the X chromosome in sperm to be of significance in X-inactivation, it is important to establish homology between the X chromosomes of eutherians, marsupials and monotreme mammals. Gene mapping in other mammalian groups has shown that many genes are conserved on the X between eutherian species, and between the human and marsupial X chromosome (Graves, 1995a, Samollow and Graves, 1998). Comparative X chromosome painting (Glas et al., 1999) confirmed that the X chromosomes are partly conserved between marsupial and eutherian mammals. At least some of these genes map to the X chromosome in monotremes (Graves and Watson, 1991). However, attempts to examine homology between the monotreme and human X by chromosome painting have not, so far, been successful.

The differentiation of sex chromosomes by degradation of the Y chromosome in mammals lead to dosage imbalance. The inactivation of one of the X chromosomes in females, results in dosage compensation; equal levels of expression of X-linked genes between females and males. As the X-inactivation system and the maintenance of the inactivation state is an important mechanism to equalize gene function, it was suggested that gene content of the X chromosome would be conserved (Ohno, 1967). If genes were moved from the X by translocations, then dosage compensation would be disrupted and have a deleterious affect.

4.1.1 The mammalian X chromosome

4.1.1.1 The eutherian X chromosome

The human X chromosome makes up 5% of the haploid genome and contains roughly 1500 genes. In other eutherians, the X makes up 5%, and gene mapping has

demonstrated a highly conserved gene content of the eutherian X chromosome (Wakefield and Graves, 1996). Almost without exception genes on the X in humans are on the X in all other eutherian mammals. For example, the gene content of the X chromosomes of mice and humans is almost entirely conserved (Wakefield and Graves, 1996). Even the order of genes has been highly conserved.

The X is completely differentiated from the Y chromosome except for two small pseudoautosomal regions (PAR). PAR 1 is 2.6Mb, contains 13 genes and lies on the tip of the short arms of the X and Y chromosome (El-Mogharbel and Graves, 2000). During male meiosis there is pairing and recombination within the PAR1. Deletion of the PAR1 results in failure of X-Y pairing and sterility (Mohandas et al., 1992). PAR2 (0.5Mb) is located at the ends of Xq and Yq. It contains four genes and occasionally pairs during male meiosis (El-Mogharbel and Graves, 2000).

4.1.1.2 The marsupial X chromosome

The basic marsupial X chromosome (eg the dunnart and wombat) is smaller than its eutherian counterpart, making up only 3% of the haploid genome. The X chromosome in macropodid marsupials (eg the tammar wallaby) is larger due to the addition of the nucleolar organizer and associated heterochromatin (Hayman and Martin, 1974).

There is no detectable PAR on the marsupial X chromosome, and no detectable pairing between the X and the tiny (10mb) marsupial Y chromosome (Sharp, 1982). Painting the dunnart X chromosome with a Y probe detects no signal on the X (Toder et al., 2000). The tammar wallaby shares a heterochromatic sequence between the short arm of the X and long arm of the Y chromosome, but no pairing occurs between these regions in male meiosis (Sharp, 1982, Toder et al., 1997).

4.1.1.3 The monotreme X chromosome

The monotreme X chromosome was recognized by its presence in one copy in males and two in females and represents 6% of the haploid genome (Watson et al., 1992). At male meiosis, the short arm of the monotreme X chromosome pairs completely with an unpaired element (known as E2), the putative Y chromosome, forming the beginning of a translocation chain.

The meiotic chain consists of 9 elements in the echidna and 8 elements in the platypus. There are two theories as to how this meiotic chain segregates during meiosis. One theory proposes that the meiotic chain works as multiple sex chromosomes, with the chain forming an XYXYXYXY in platypus and a XYXYXYXYX in echidna (Bick,

1992). In this case all the X chromosomes would go to one pole and all the Y chromosomes to the other. In platypus one type of sperm would contain 4 X chromosomes and the other 4 Y chromosomes, and in echidna one type of sperm would contain 5 X chromosomes and the other 4Y chromosomes. Each would fertilize an XXXX egg.

The other theory proposes that there is an X and Y at the front of the platypus and XYX in the echidna chain that pair with translocated autosomes. In this case the two types of gametes formed in echidna are XXqsu and Yprt. Therefore the male gamete containing XXqsu would bind with the female gamete XXprt and the male gamete Yprt would merge with the XXqsu containing egg. The zygote must have one copy of pqrstu, otherwise the zygote would lack DNA. Therefore there are two lethal combinations XXXXqqssuu and XXYpprrtt (Watson et al., 1992). The idea that there is one type of sperm for one type of egg is an intriguing idea.

The first theory predicts that the female has no unpaired elements; however, platypus and echidna do have the same unpaired elements in both sexes (Watson et al., 1992), and therefore females as well as males would have a meiotic chain, suggesting the theory of multiple sex chromosomes is incorrect.

4.1.2 Gene mapping on the mammalian X chromosome

Mapping genes on the X chromosomes of eutherians, marsupials and monotremes showed that genes on the long arm and the pericentromeric region of the short arm of the human X are conserved between the three different mammalian groups. This X chromosome conserved region (XCR) delineates an ancient X at least 170 million years old (reviewed by Graves, 1995a). The eutherian X chromosome can be divided into two different regions.

The X conserved region in marsupials (on the long arm of the tammar X) contains the human Xq genes *G6PD, F8, F9, GLA* and *AR* (Spencer et al., 1991b, Wilcox et al., 1996) (figure 4.1, see table 4.1 for gene names). The long arm of the tammar wallaby X chromosome is hypoacetylated, suggesting that it is inactivated and contains an X inactivation centre (XCI) (Wakefield et al., 1997). There is a corresponding YCR that contains several genes shared with the marsupial X - chromosome, implying that the chromosomes are monophyletic.

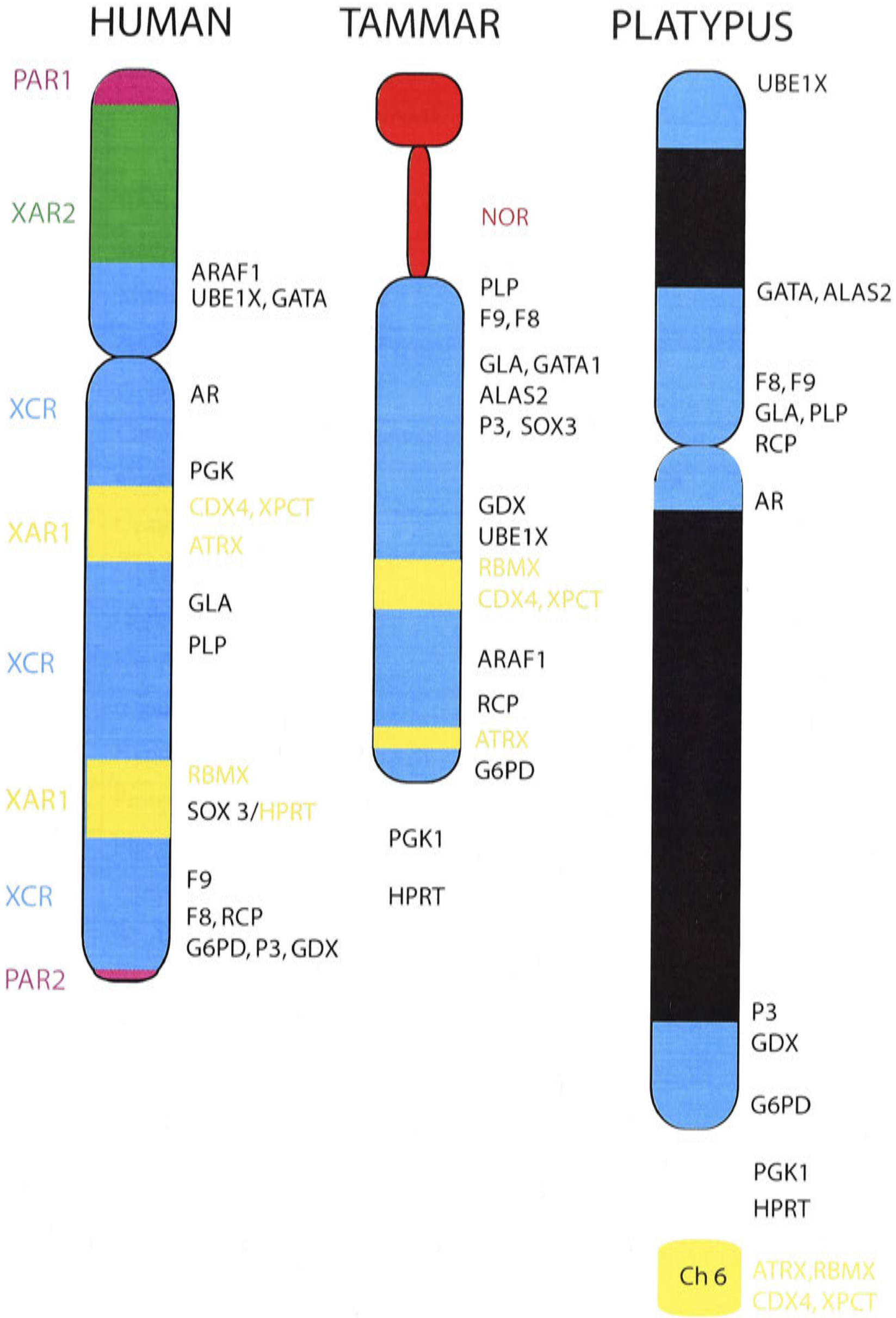


Figure 4.1: Gene mapping on the X chromosome in the human, tammar wallaby, and platypus. Light blue region is the conserved region of the X chromosome (XCR). Yellow represents a region (XAR1) added 130-170mya, the green represents a region (XAR2) added 80-130mya (Samollow & Graves, 1998; Waters, 2002; Wilcox, 1996).

Table 4.1: Eutherian X chromosome genes

LOCUS	LOCUS NAME	HUMAN LOCUS POSITION
ALAS2	Erythroid 5-amino-levulinate synthase	Xp11.21
AR	Androgen receptor	Xq11.2-q12
ARAF1	Murine sarcoma oncogene	Xp11.4-p11.21
ATRX	α-thalassemia and mental retardation-like gene on the X	Xq13.1-21.1
CDX4	Caudal type homeo box transcript	Xq13.2
F8	Coagulation factor VIII	Xq28
F9	Coagulation factor IX	Xq27.1-q27.2
G6PD	Glucose-6P dehydrogenase	Xq28
GATA1	Globin transcription factor	Xp11.23
GDX	Ubiquitin like protein 4	Xq28
GLA	α-galactosidase	Xq21.3-q22
HPRT	Hypoxanthine phosphoribosyltransferase	Xq26.1
P3	Protein P3	Xq28
PGK	Phosphoglycerate kinase	Xq13.3
PLP	Proteolipid protein	Xq21.33-q22
RBMX	RNA-binding domain on the X	Xq26
RCP	Red cone pigment	Xq28
SOX3	SRY like-HMG box	Xq26-q27.1
XPCT	Solute carrier family 16 (monocarboxylic acid transporter	Xq13.2
UBE1X	Ubiquitin activated enzyme on the X	Xp11.23

Mapping genes on the short arm of the human X distal to Xp11.23 in marsupials and monotremes established a region that was added to the eutherian X. A human Xp gene, *OTC* (ornithine transcarbamylase), was found to map to the short arm of chromosome 5 (5p) in the tammar wallaby and was also autosomal in the dunnart. It therefore did not belong to the conserved mammalian X chromosome (Sinclair et al., 1987). A second gene not conserved between sex chromosomes of mammals was *ZFY* (zinc finger protein). This gene was originally claimed to be the mammalian testis determining factor, but its autosomal location in marsupials disqualified it from this role (Sinclair et al., 1988).

Other genes from the human Xp also mapped to autosomes in marsupials, and also in monotremes (Spencer et al., 1991a, Watson et al., 1991), defining a region (XAR) added to the eutherian X after the divergence of marsupials 130mya, but before the eutherian radiation 80mya. This XAR corresponds to a Y added region that constitutes most of the human Y (Waters, 2002).

There is now a second region believed to have been added to the eutherian X (Waters, 2002) in a marsupial/eutherian ancestor after the divergence of monotremes (130-170mya). It was found that several genes mapping to the X chromosomes in both marsupials and eutherians did not map to the X chromosome in monotremes. These genes include *ATRX, RBMX* and *CDX4*, which mapped to platypus chromosome 6 (Waters, 2002), and *HPRT* and *PGK,* which cell hybrid analysis demonstrated, were excluded from the X in platypus (Wrigley and Graves, 1988a).

In total 9 human X chromosome genes have been mapped to 5p in tammar wallaby, and three genes have been mapped to chromosome one in the tammar wallaby. The cluster found on 5p in tammar wallaby is also found on 3q in the dunnart suggesting the addition involved one large rearrangement. Mapping in monotremes is important to provide an outgroup to show that the XAR region in eutherians was an addition to the eutherian X, not loss from the marsupial X.

Comparative gene mapping therefore led to the formation of the addition/attrition hypothesis (Graves, 1995b). The mammalian sex chromosomes were originally a homologous autosome pair. In a mammalian ancestor, a mutation produced new male determining genes on one of these homologues. Accumulation of male-advantage genes on this proto-Y led to suppression of recombination in order to protect an association between sex determining and differentiating genes. This loss of

recombination may have occurred in stages corresponding to major inversions of the Y, within which gradual degradation of the Y chromosome occurred. Once a region had been added to the sex chromosomes, it was exposed to the selective pressures leading to the degradation and rearrangements of the YAR (Y chromosome added region). Once the YAR genes had been lost, their partners would have been recruited to the X-inactivation system to ensure expression balance between males and females (Graves et al., 1998).

4.1.3 X chromosome inactivation

X-inactivation is initiated and maintained through several different mechanisms (section 1.5.4). The initial step for inactivating the X chromosome is the expression of *XIST*, which covers the X chromosome. It is still unknown how the *XIST* transcript is propagated over the X chromosome. It has been proposed that LINE-1 elements on the X chromosome may act as booster elements helping to extend the *XIST* transcript over the inactive X chromosome (Lyon, 1998, Riggs et al., 1985). After *XIST* has covered the X, DNA methylation, histone deacetylation and MacroH2A localization to the inactive X occur helping to maintain the inactive state of the chromosome.

4.1.3.1 Booster elements in mammalian X-inactivation

LINE-1 (L1) is a retrotransposon that is copied and pasted throughout the human genome and accounts for 15 to 17% percent (Kazazian, 2000). L1 consists of a 5' promoter and two open reading frames, ORF1, which produces a nucleic acid binding protein, and ORF2, which produces a protein with endonuclease, reverse transcriptase, and zinc knuckle domains (Kazazian, 2000). L1 elements are flanked by short duplications of genomic DNA that are target sites for retrotransposition.

An L1 probe hybridized to human metaphases showed a concentration of signal in G-bands of autosomes. However, L1 sequences were found throughout the X chromosome, the L1 probe hybridizing along the entire length of the X chromosome (Boyle et al., 1990). Unusually strong clustering of L1 elements is found at Xq13 (the location of the XIC) and Xq21, where 45% and 39% of sequences corresponded to L1 repeats. This distribution of L1 is significantly higher than that of other X chromosome repetitive sequences (Bailey et al., 2000, Boyle et al., 1990). Additional L1 clusters were observed at Xq22, Xq24-25 and Xq27.

In the mid nineteen eighties it was proposed that way-stations on the X chromosome allowed the transmission and elongation of the X-inactivation signal along the X chromosome (Riggs et al., 1985). In 1998, Mary Lyon proposed that LINE-1 repeats might act as these way stations transmitting the X-inactivation signal along the X chromosome (Lyon, 1998). Evidence for this hypothesis was the high level of LINE-1 repeats on the X chromosome and transgenic experiments involving the integration of XIC onto an autosome.

The X chromosome has a L1 content of 26%, whereas autosomes have an average L1 content of 13.4%, suggesting that the X chromosome has been preferentially targeted for L1 retrotransposition or that accumulation of L1 has been selected for (Bailey et al., 2000). The concentration of L1 elements on the X led Lyon to propose that they were the 'way stations' predicted by Riggs (1985) to help transmit the X-inactivation signal along the X (Lyon, 1998).

L1 elements can be classified into younger and older LINE sequences. The older elements (L1M4 and LIM2) show only slight enrichment on the X chromosome, whereas the younger elements (L1M1, L1P5 to L1P1) are significantly over-represented on the X chromosome (Bailey et al., 2000). The L1M1 and L1P4 subclasses of LINE-1's were active in the eutherian to prosimian lineage between 60-100 million years ago. Therefore younger L1 sequences may play an important role as "booster" elements that promote X-inactivation and have a stabilizing effect in maintaining X-inactivation.

In XIC transgenic experiments the stability of inactivation of an autosome correlates with cytogenetic banding and L1 density (Lyon, 1998). For example, a XIC transgene integrated into the pericentromeric region of chromosome 12, showed all the signs of inactivation of genes on the proximal region of chromosome 12 (Lee and Jaenisch, 1997). There was evidence of hypoacetylation and *Xist* RNA over the proximal end of chromosome 12. However, the distal end of the chromosome showed none of these inactivation characteristics. This corresponded with the distribution of L1 over the proximal region of chromosome 12, but not at the distal end. According to the L1 booster hypothesis, X-inactivation did not spread to the distal region, as no "booster" elements were present to promote extension of inactivation along the distal end of the chromosome (Lyon, 1998).

4.1.3.2 Marsupial and monotreme X-inactivation

Marsupials have a late replicating X chromosome (Graves, 1967) that is always paternally derived (Cooper et al., 1983, Sharman, 1971). Paternal X-inactivation occurs

in all tissues of marsupials, in contrast to the eutherian XIC, which is random except in rodent extraembryonic tissue (Takagi and Sasaki, 1975). The occurrence of imprinted X-inactivation in eutherians as well as marsupials suggests that it represents the ancestral form of X-inactivation (Cooper, 1971).

X-inactivation was demonstrated in marsupials using enzyme assays to study isozyme levels of X-linked genes in heterozygotes (Cooper et al., 1993). X-inactivation in marsupials is not as stable, with G6PD, PGK-A, and GLA paternally inactivated in blood but showing different patterns of inactivation in other tissues (reviewed by VandeBerg et al., 1987). HPRT is also paternally inactivated in lymphocytes and fibroblasts (Graves and Dawson, 1988). Marsupial X-inactivation also differs from that of eutherians in that most marsupial tissues have no heterochromatic body at the periphery of the nucleus (McKay et al., 1987). Digestion of marsupial chromosomes with Dnase 1 and Msp 1 showed no differential condensation between the active and inactive chromosomes (Loebel and Johnston, 1993), whereas the same technique in eutherians showed a lower sensitivity in the inactive X chromosome (Kerem et al., 1983).

Methylation of CpG islands on the eutherian inactive-X chromosome stabilizes X-inactivation. However, no differential methylation of CpG islands was revealed between alleles on the active and inactive X chromosome in marsupials (Kaslow and Migeon, 1987, Piper et al., 1993). This suggests a role in maintenance and stabilization rather than initiation of X chromosome inactivation. Although G6PD is completely inactivated in marsupial lymphocytes, bisulfite sequencing showed hypomethylation 5' of CpG islands of both inactive paternal and active maternal G6PD alleles. As for eutherians, methylation was observed in many internal regions of genes on the active X chromosome (Migeon et al., 1989, Piper et al., 1993).

A feature conserved between eutherians and marsupials is the hypoacetylation of the inactive X chromosome. H3ac and H4ac antibodies were used to detect acetylated histones in female tammar metaphase spreads, demonstrating the hypoacetylation of the long arm of one X chromosome, as well as the heterochromatic short arm of both X chromosomes (Wakefield et al., 1997).

X-inactivation has not yet been conclusively demonstrated in monotremes. There is asynchronous replication of the X chromosomes in female cells, but it was confined to Xp (Murtagh, 1977, Wrigley and Graves, 1988a). As the short arm of the X pairs with element E2 in the meiotic chain at male meiosis, there should be no need for

dosage compensation in this region. Recently, *G6PD* which maps to the long arm of the platypus X chromosome, was found to show equal levels of expression in male and female cells, suggesting that dosage compensation occurs in platypus (J.Deakin, personal communication).

4.1.4 Comparative painting in this study

The platypus X chromosome has been identified by the observation of one chromosome in males and two chromosomes in females (Wrigley and Graves, 1988a) and shown to share at least some genes with the human X (Watson et al., 1990). To assess the significance of my finding that the conserved mammalian X chromosome had a conserved position in mammalian sperm, I used comparative chromosome painting to directly demonstrate conservation between the platypus X chromosome and the human X chromosome. The successful hybridization of chromosome probes depends on the chromosome homology of the species being studied. For example, cross species hybridizations between eutherian chromosomes, cross species hybridization between marsupial chromosomes, or cross species hybridization between bird chromosomes is relatively easy. However, cross species hybridizations between more distantly related animals are incredibly hard. The successful hybridization of the tammar wallaby X chromosome to the human X chromosome was considered a miracle (Glas et al., 1999), and the cross hybridization of chicken chromosomes to turtle chromosomes was considered breaking the ZOO-FISH barrier (Graves and Shetty, 2000).

In collaboration with Patrick Kirby, I painted the platypus X chromosome onto human metaphase spreads, discovering a region conserved between the platypus X and the human X chromosomes.

4.2 Results

A male platypus fibroblast cell line was sent to Professor M.A. Ferguson-Smith's laboratory in Cambridge University, U.K, where the chromosomes were flow-sorted. Chromosomes were divided into different peaks, each representing a specific chromosome. In this study peak 7 (whole X chromosome), peak 13J (E2) and peak 18 (chromosome 22) were used in cross species hybridizations with human metaphase spreads.

4.2.1 MON1 repeats

Cross-species painting, as well as gene cloning and FISH, are difficult in monotremes because of the high content of repeat sequences. One of the most prevalent is MON1 (Gilbert and Labuda, 1999). One of the problems encountered when using chromosome painting was the hybridization of our paints to these repeats, especially after two or three DOP-PCR amplifications. The additional rounds of DOP-PCR seemed to amplify the MON1 repeats preferentially (figure 4.2). Work done in collaboration with Patrick Kirby (Research School of Biological Sciences at the Australian National University), showed that MON1 is a large 254bp repeat. We used DOP-PCR and biotin labeling to observe the physical location of the repeat. The MON repeat was found mostly at the ends of the larger chromosomes, including the X where it hybridized to the distal region of both arms (figure 4.3).

Amplified paints containing MON1 repeats produced a high background, making any experimental results useless, especially when trying to interpret X chromosome conservation (figure 4.2). Therefore to avoid a high content of MON1 repeat in the paint, only paints derived from the primary or secondary DOP-PCR were used.

4.2.2 Hybridization with Platypus X and E2 paints

4.2.2.1 Hybridization to platypus chromosomes

It was important to test the quality of the paints, as it is extremely hard to cross-hybridize between two species that diverged over 170mya. Peak 7 (the platypus X chromosome) and 13J (element E2, the putative Y chromosome) were therefore hybridized onto platypus metaphase spreads.

Peak 7 hybridized to one large submetacentric chromosome with the size and morphology of the platypus X, and also to an unpaired element in males. In female cells, this paint hybridized to a pair of large submetacentrics with the size and morphology of the platypus X (figure 4.4a). Peak 13J hybridized to the E2 element and to the short arm of the chromosome identified as the platypus X in male derived cells and to the short arms of the two X chromosomes in females (figure 4.4b and 4.5). This confirms the homology between Xp and E2 deduced from the meiotic pairing.

4.2.2.2 Hybridization of platypus X and E2 paints to human chromosomes

Stringent controls need to be used when painting between two distantly related species, to ensure any signal found is not an artefact. Each experiment also had a

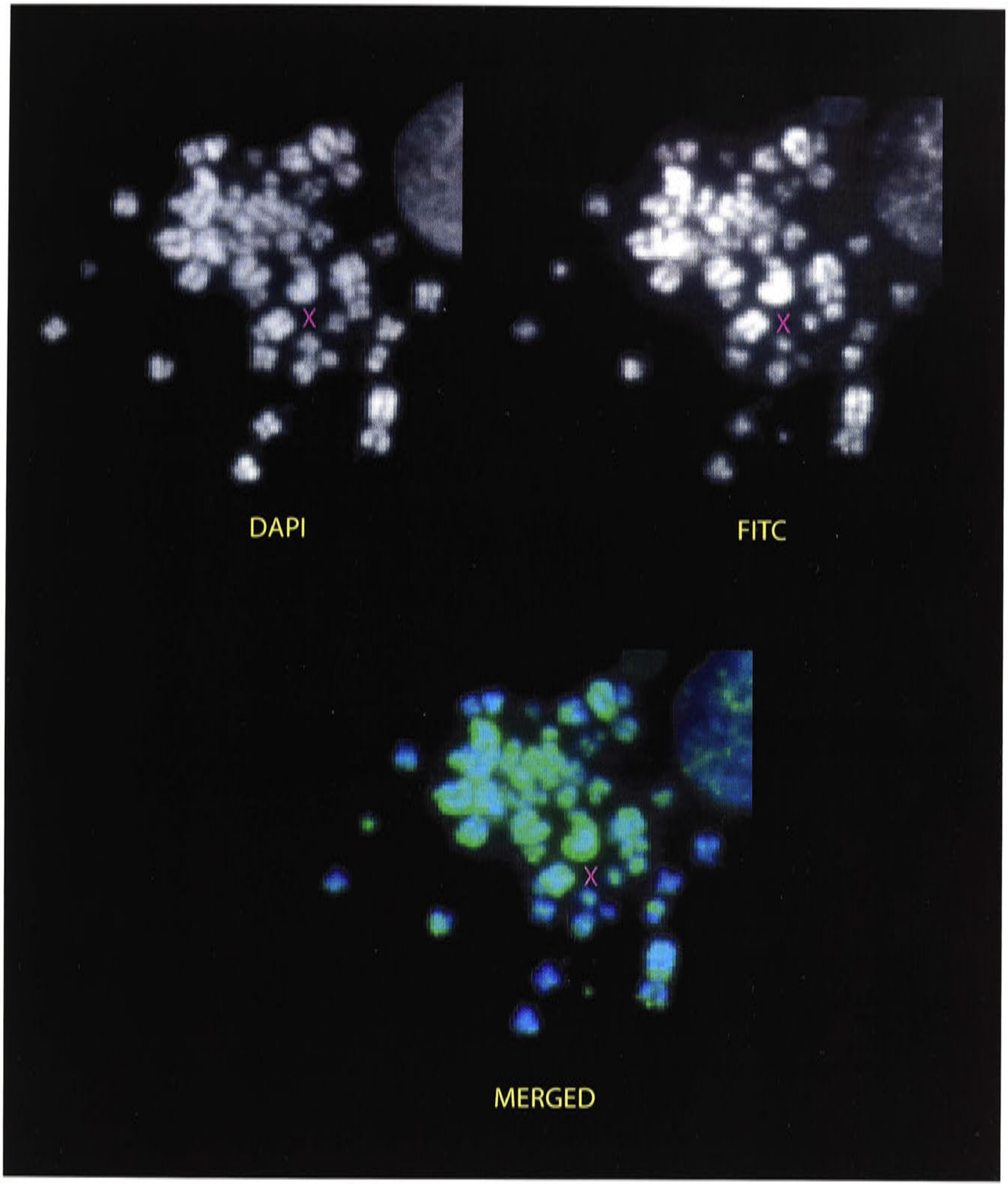


Figure 4.2: DOP-PCR amplification of MON1 repeat in platypus paints. Peak 7 hybridized to platypus metaphase after two rounds of DOP-PCR. Shows signal still hybridized to X, but also to the MON repeats, which make it impossible to distinguish E2.

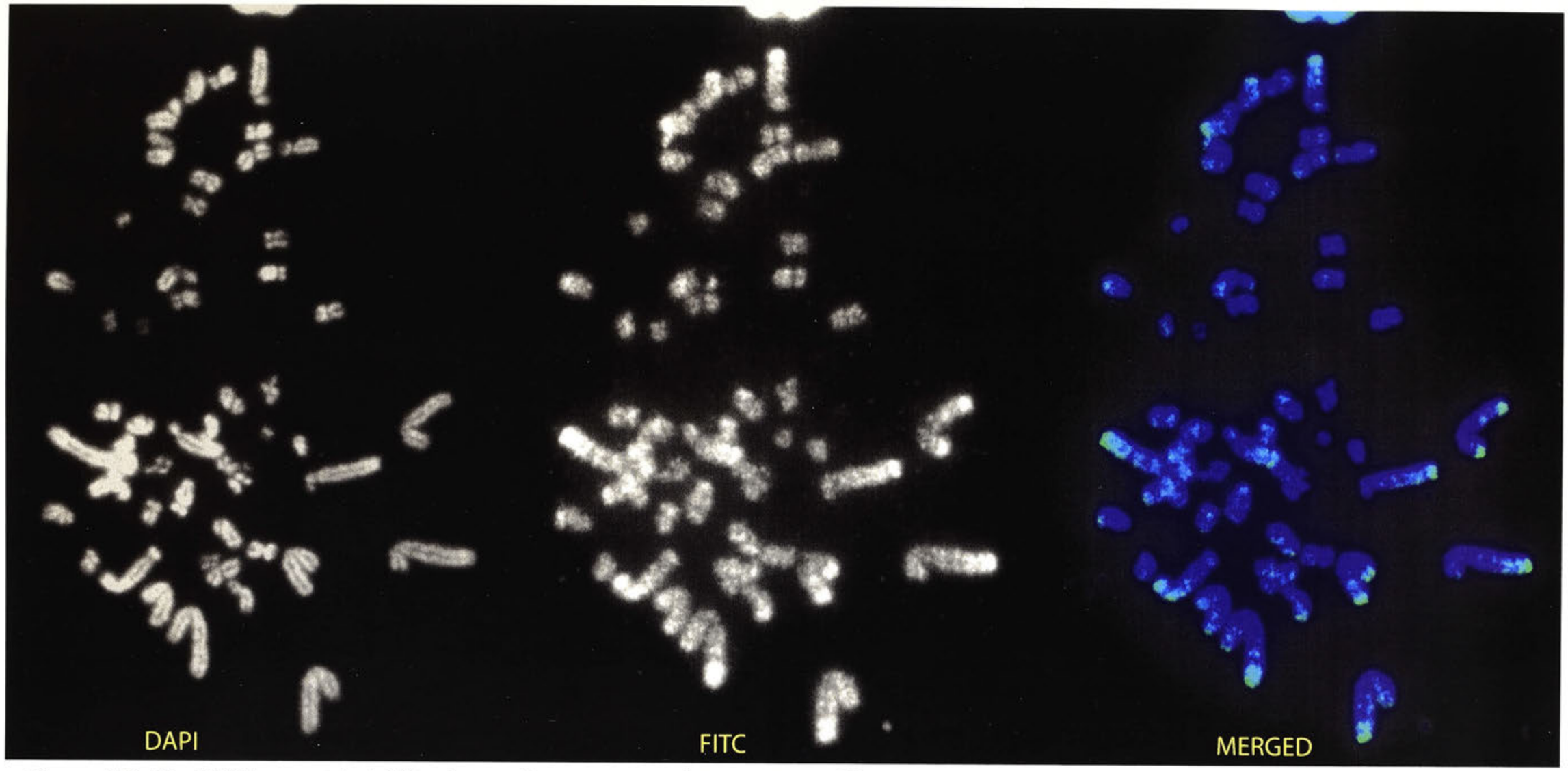


Figure 4.3: The MON repeat hybridized to a platypus metaphase spread. (Shown stained with DAPI, labeled with FITC to reveal position of the MON1 probe, and a merged image) Most of the repeat was found clustered at the ends of the 6 large platypus chromosomes.

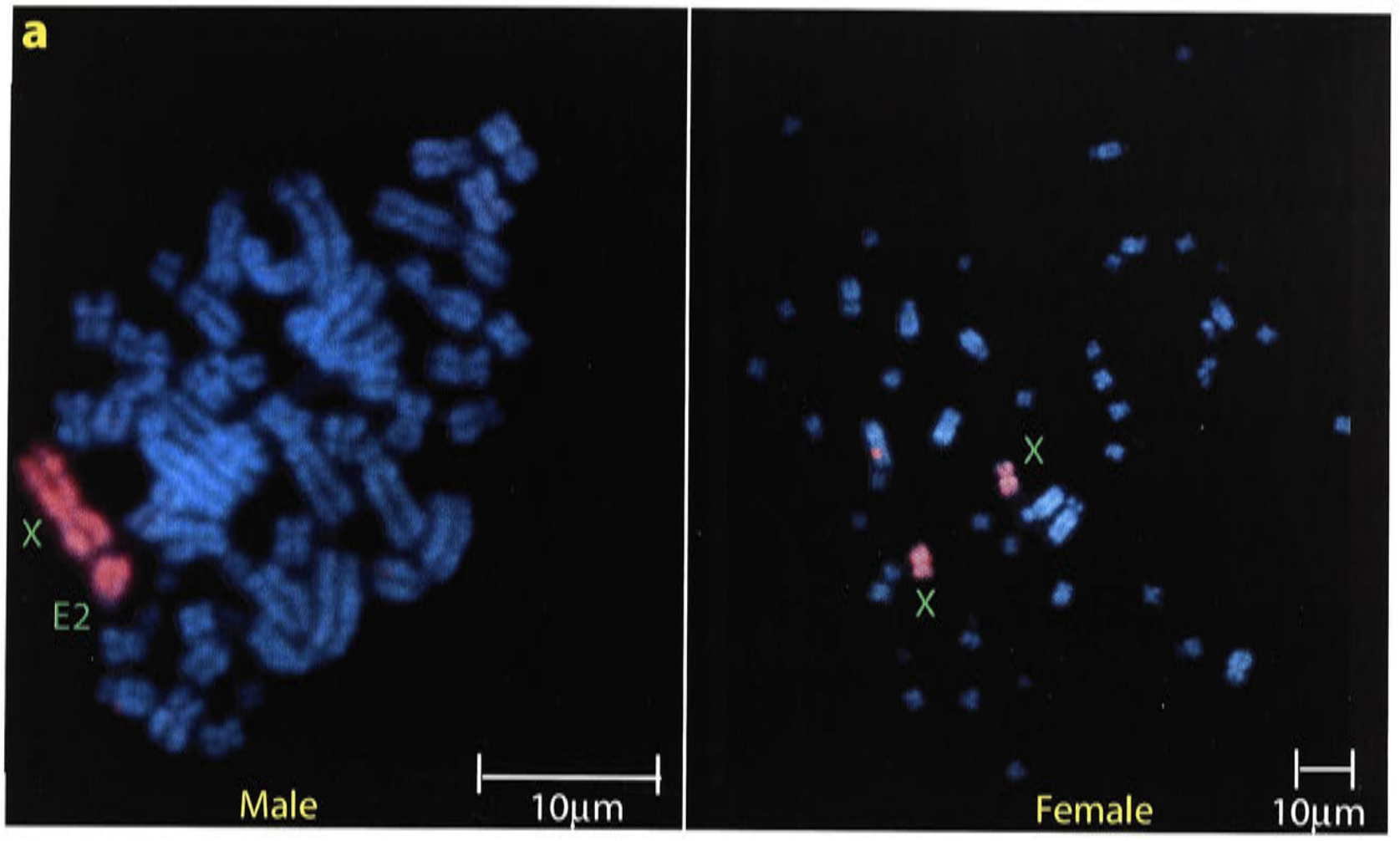


Figure 4.4: Testing platypus flow-sorted chromosomes. (a) The whole X chromosome paint (peak 7) hybridized to the X and E2 in males, and to the pair of X chromosomes in females. (b) The E2 paint (peak 13J) hybridized to Xp and E2 in males and to the short arm of both X chromosomes in female cells.

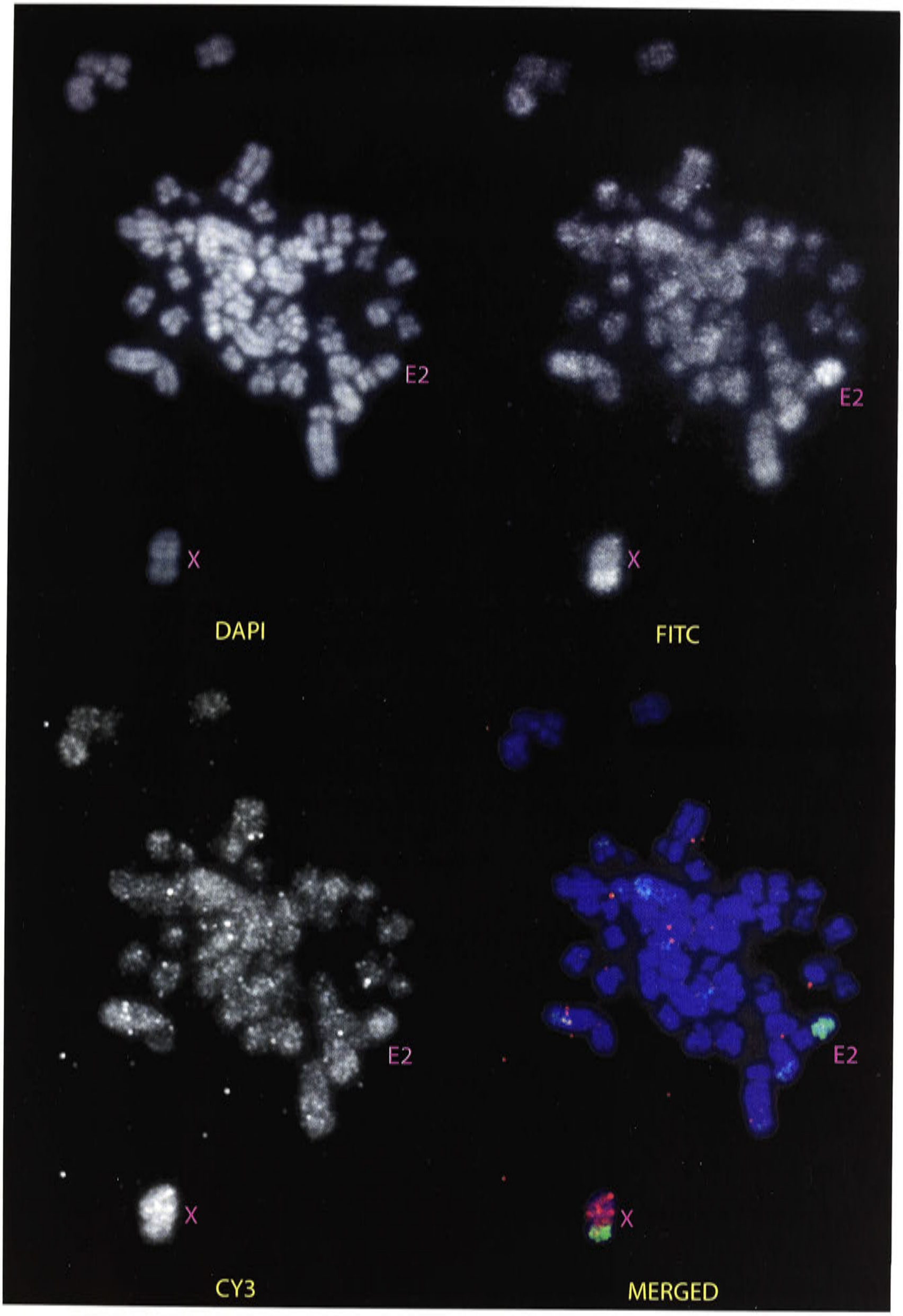


Figure 4.5: Double painting with the platypus X and E2 chromosome paints onto a platypus metaphase spread (DAPI stained). E2 (peak 13J labeled with FITC) hybridized to E2 and Xp, and the whole X (peak 7 labeled with Cy3) hybridized to E2 and all of the X chromosome.

negative control, where only the secondary antibody was used to make sure any signal present was not due to non-specific binding of the antibodies. Autosome platypus paint was also hybridized to human metaphase slides to compare with any signals obtained with the X chromosome to test if the signal was due to X specific or repetitive (eg. MON1) platypus sequences.

The E2 (platypus Xp) paint (Peak 7) was hybridized to 46 human metaphase spreads over 5 days. After washing a signal was consistently observed on a chromosome with the size and morphology of the X chromosome. To ensure that the signal was hybridizing to the human X chromosome, the paint was hybridized to male and female human metaphases. On male metaphases one signal was observed, whereas in females two signals were observed (figure 4.6). Although a consistent signal was observed on the X chromosome in all metaphase spreads, the length of the signal varied between spreads and preparations (figure 4.7a). The signal was always observed on Xq and was always strongest just below the centromere. However, in 10% of metaphases the signal was observed from proximal Xp down to the distal end of Xq.

The whole platypus X chromosome paint was hybridized under the same conditions. In male metaphases one signal was observed, whereas 2 signals were observed in female metaphases on a chromosome with the size and morphology of the X (figure 4.8). The length of signal again varied between different metaphase spreads. 90% of these signals were restricted to a region just below the X chromosome, but 10% of signals were observed from proximal Xp down to the distal end of Xq (figure 4.7b). The variation in hybridization to the X chromosome did not differ with either the X chromosome or the E2 paint.

An X chromosome paint that had undergone more than two DOP-PCR amplifications was found to hybridize to numerous human centromeres, including that of the X chromosome (figure 4.9).

4.2.3 Platypus chromosome 17-21 autosome paint

4.2.3.1 Hybridization of an autosome to platypus chromosomes

Peak 18, derived from a small (pair 17-21) submetacentic autosome, was first painted to platypus chromosomes to test the quality of the paint before hybridizing it to human chromosomes. This paint produced signal specifically on a small pair of submetacentric platypus chromosomes (figure 3.15)

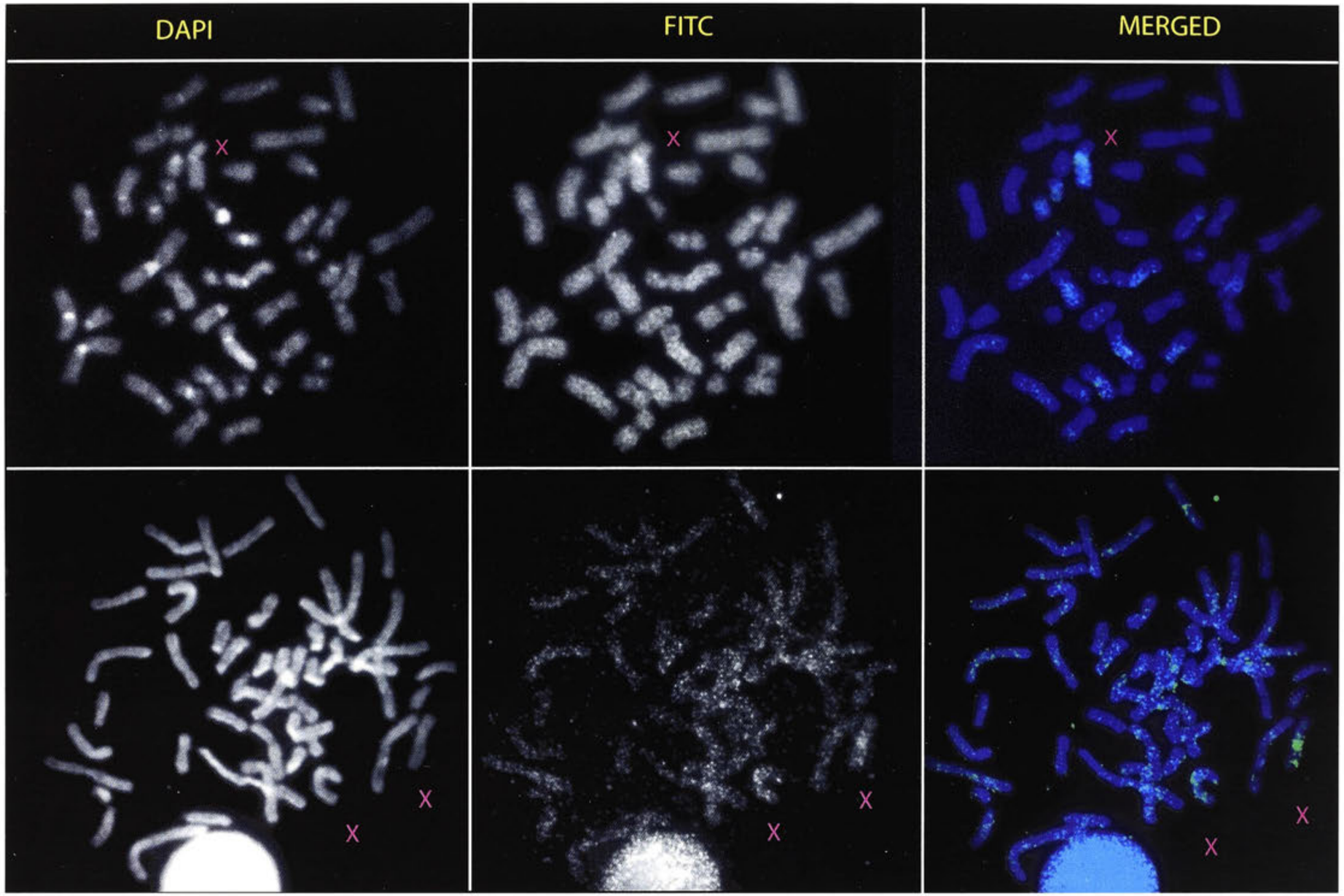
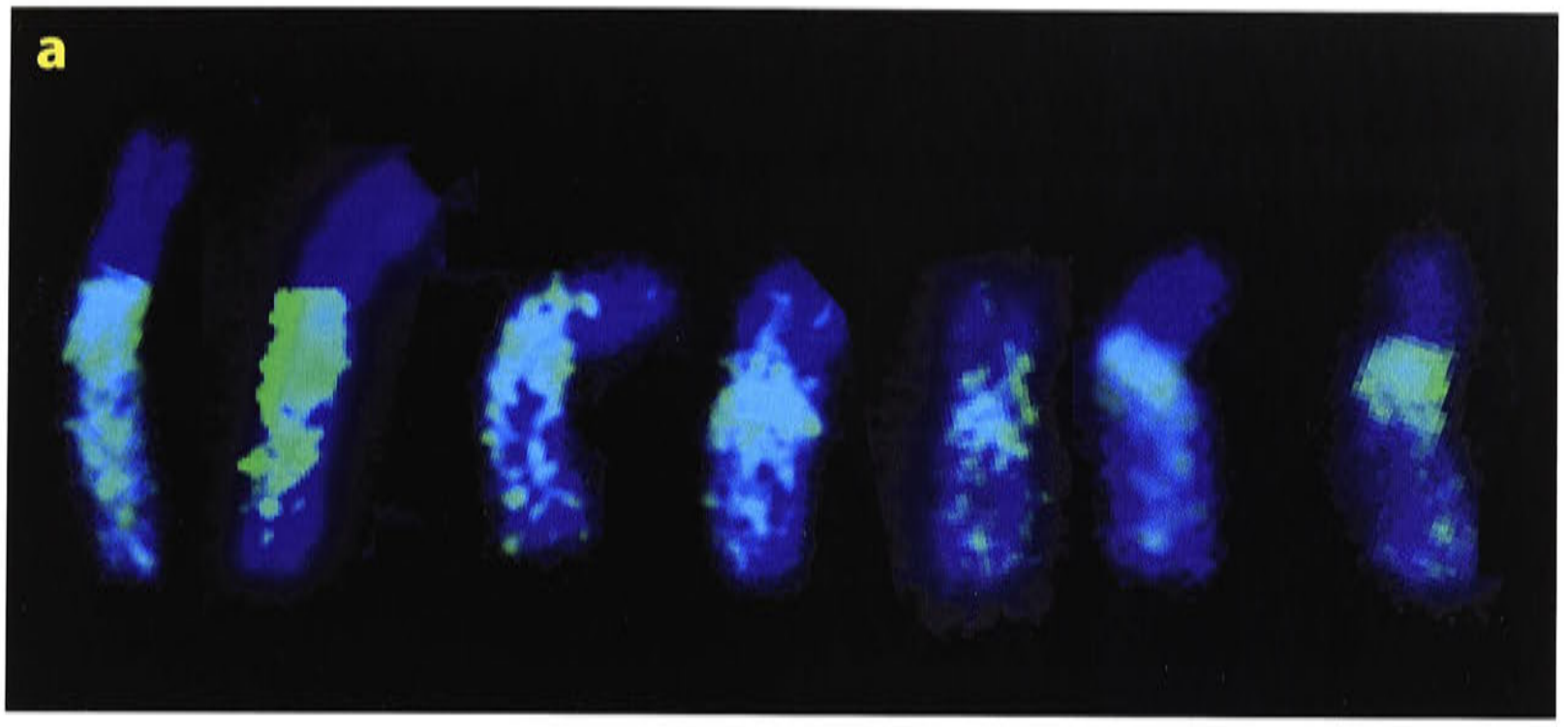


Figure 4.6: Cross species painting of playpus E2 chromosome paint (Peak 13J) onto a human metaphase spread. A consistent signal was observed on the single male X chromosome and on both female X chromosomes.



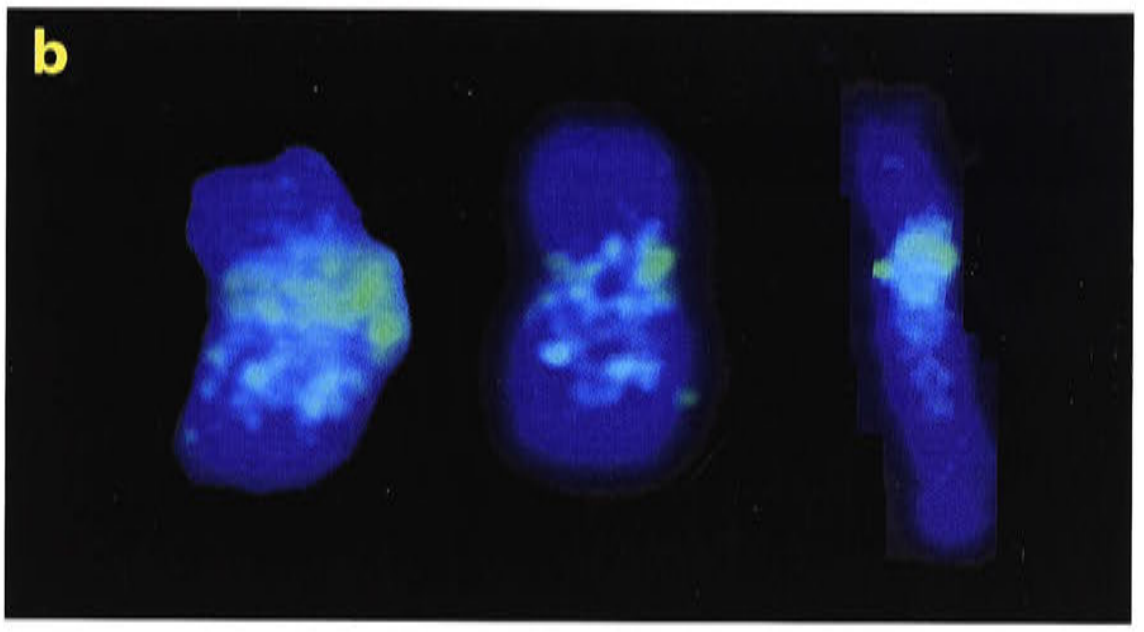


Figure 4.7: Different variations in the extent of hybridization of the E2 probe (a) and the whole X chromosome probe (b) to the human X chromosome. Different chromosome sizes reflect different levels of condensation of the metaphase spreads. In some spreads two thirds of the X chromosome (including the whole of Xq and proximal Xp hybridized, whereas in other spreads only the region below the centromere hybridized.

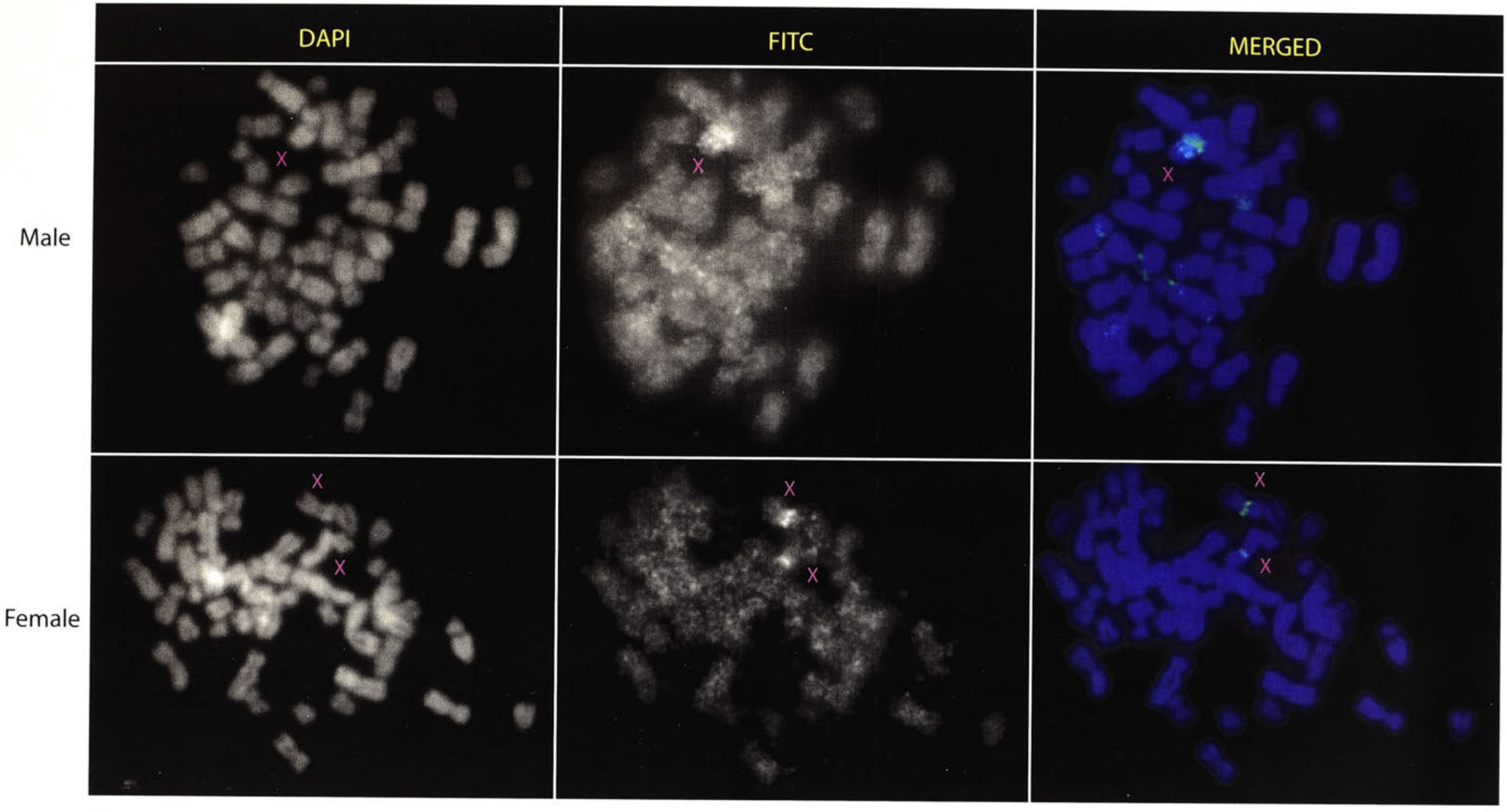


Figure 4.8: Cross species painting of playpus whole chromosome paint (Peak 7) onto a human metaphase spread. A consistent signal was observed on the single male X chromosome and on both female X chromosomes.

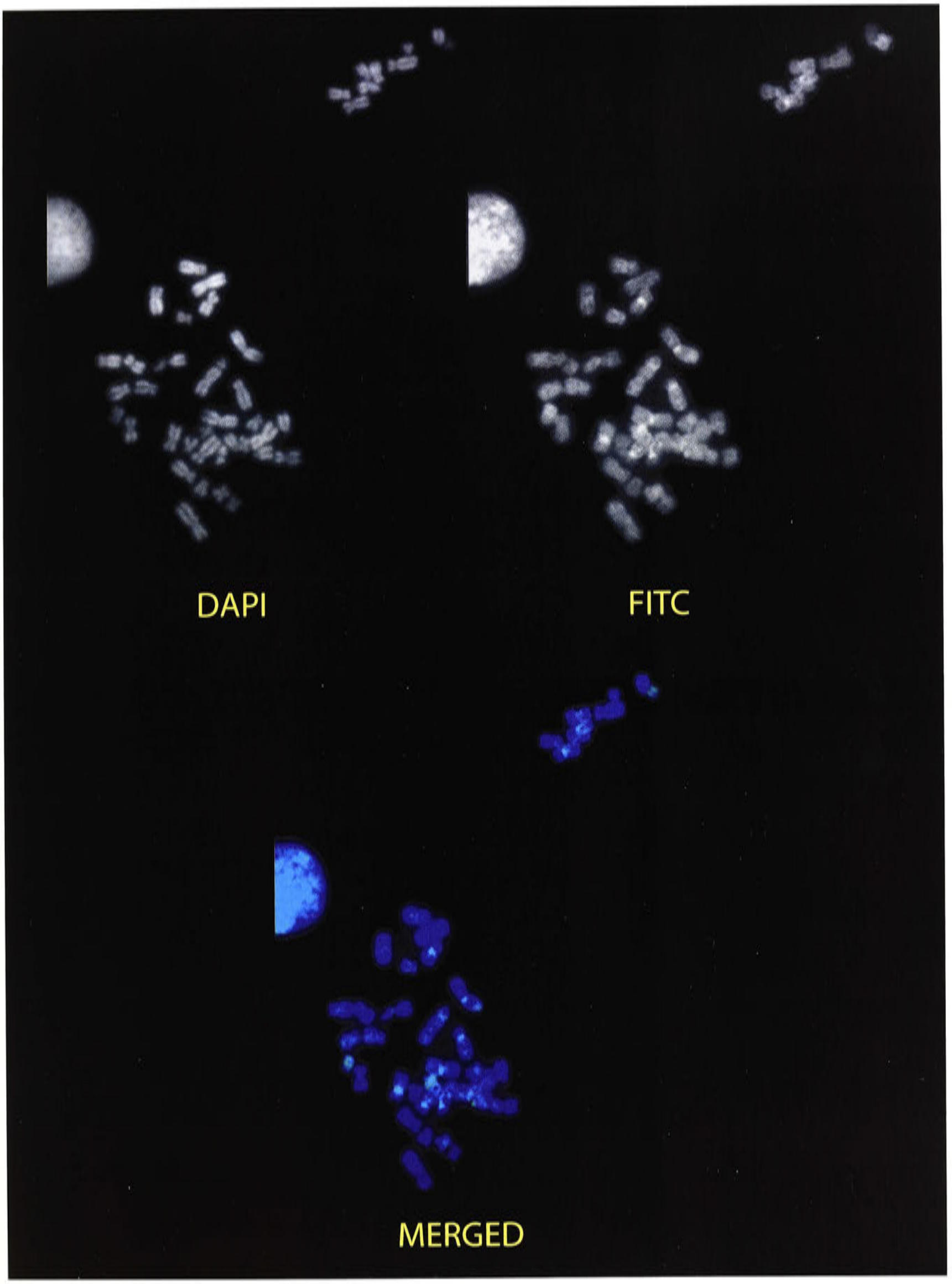


Figure 4.9: DOP-PCR problems in cross-species painting. Peak 13J after two rounds of DOP-PCR. Though humans do not contain the MON1 repeat, non-specific binding to centromeres of several human chromosomes was observed, probably as a result of preferential MON1 amplification.

4.2.3.2 Hybridization of an autosome to human chromosomes

The signal observed on the human X chromosome could be due to conserved X specific sequences or alternatively, be a byproduct of repetitive sequences in the paint. If the signal was really due to conserved sequences between the platypus and the human X chromosome, then the autosome paint should not hybridize to the same region of the X. To test the specificity of the signal observed in humans with the platypus X and E2 paints, this autosome paint was therefore hybridized to human chromosomes.

The autosome paint hybridized to the centromeres of a small human autosome (tentatively no. 16) and also to the proximal region of Xq (figure 4.10). The signal on the X chromosome was not detected in every spread and was not as strong as the hybridization signals observed with peak 13J and peak 7. The signal never extended past the proximal region of Xq.

4.3 Discussion

The hybridization of the platypus X chromosome paint to the human X was attempted in order to confirm the conservation of the mammalian X chromosome. Cross-species painting across a 170 million year evolutionary divide was considered unlikely to succeed, but the success of marsupial – human X cross-hybridization was encouraging.

A clear signal was detected on the human X chromosome. In some experiments the signal covered the human Xq down to near the distal end, whereas in other cells the signal localized to a broad band most intense just below the centromere. This position corresponded roughly with human Xq13-21, close to *XIST*.

The signal observed in this study was identical to that observed when the tammar X chromosome was hybridized to human chromosomes (Glas et al., 1999). There are three explanations possible for the signal observed on the human X chromosome with the platypus X paint:

1) The signals observed are a DOP-PCR artefact.
2) The platypus paint contains many unique sequences, including coding sequences conserved on the mammalian X.
3) The platypus X (and E2) paints contain conserved X-specific repetitive sequences involved in X-inactivation.

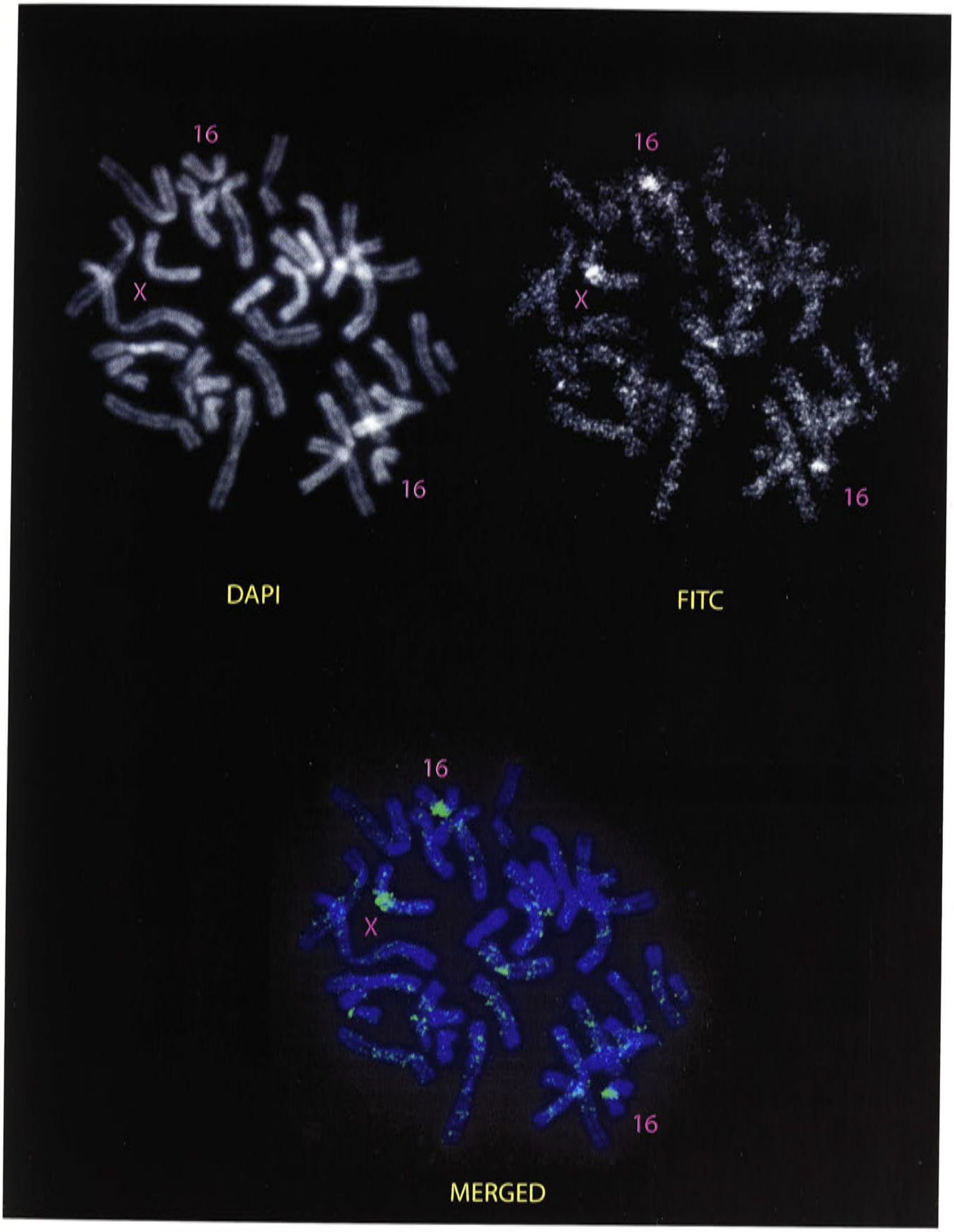


Figure 4.10: Platypus autsome painted onto a human metaphase spread. A paint (peak 18) derived from a small platypus autsome 17-21 was hybridized to the centromere of a small human autosome (tentatively no. 16) and to the proximal region of the Xq in a human male metaphase spread.

4.3.1 DOP-PCR product

DOP-PCR is used to amplify all chromosome sequences, in order to produce enough sequence for a paint. Both unique sequences and repetitive sequences are amplified in the reaction, but during successive rounds of DOP-PCR repetitive sequences are disproportionately amplified. The signal observed may, therefore, represent repetitive DNA sequences found throughout all mammals that have no functional significance. This is suggested by the hybridization of a paint subjected to multiple rounds of DOP-PCR hybridizing to the X chromosome and centromeric regions of several chromosomes (figure 4.9).

4.3.2 Does painting reveal the ancient mammal X?

The simplest interpretation of the hybridization of the platypus X chromosome to most of the human Xq, is that the X conserved region (XCR) contains conserved unique sequences shared by the platypus and human X that were part of the ancient X (170 million years old). This was the interpretation of similar results obtained by painting the tammar X onto the XCR of the human (Glas et al., 1999). This region of the human X chromosome contains about 1000 genes and includes the X-inactivation centre. The fact that E2 (peak 13J) also paints the XCR in humans would suggest that some of the ancient conserved part of the mammalian chromosome is found on Xp in platypus.

If the signal were indeed due to conserved DNA sequences, then we would expect the patterns of hybridization to reflect the pattern of gene conservation. The whole platypus X chromosome paint must contain conserved genes mapped to the platypus X; *GLA, PLP, F8, F9, RCP, GATA1, ALAS2, AR, UBE1X, P3, GDX* and *G6PD* (table 4.1). Therefore, a signal should have been observed above and below the centromere from Xp11.3 to Xq11.2, Xq21.3 and at the terminal region of human Xq from Xq26.3 to Xq28 of the human X chromosome (figure 4.11a). However, the middle region of Xq should show no signals of hybridization, since we now know that the region containing *PGK, HPRT, ATRX, RBMX* and *SOX3*, are not located on the platypus X chromosome (figure 4.11a).

The platypus E2 paint should produce a somewhat different pattern, since it contains the conserved genes *GLA, PLP, F8, F9, GATA1, ALAS2, RCP* and *UBE1X.*

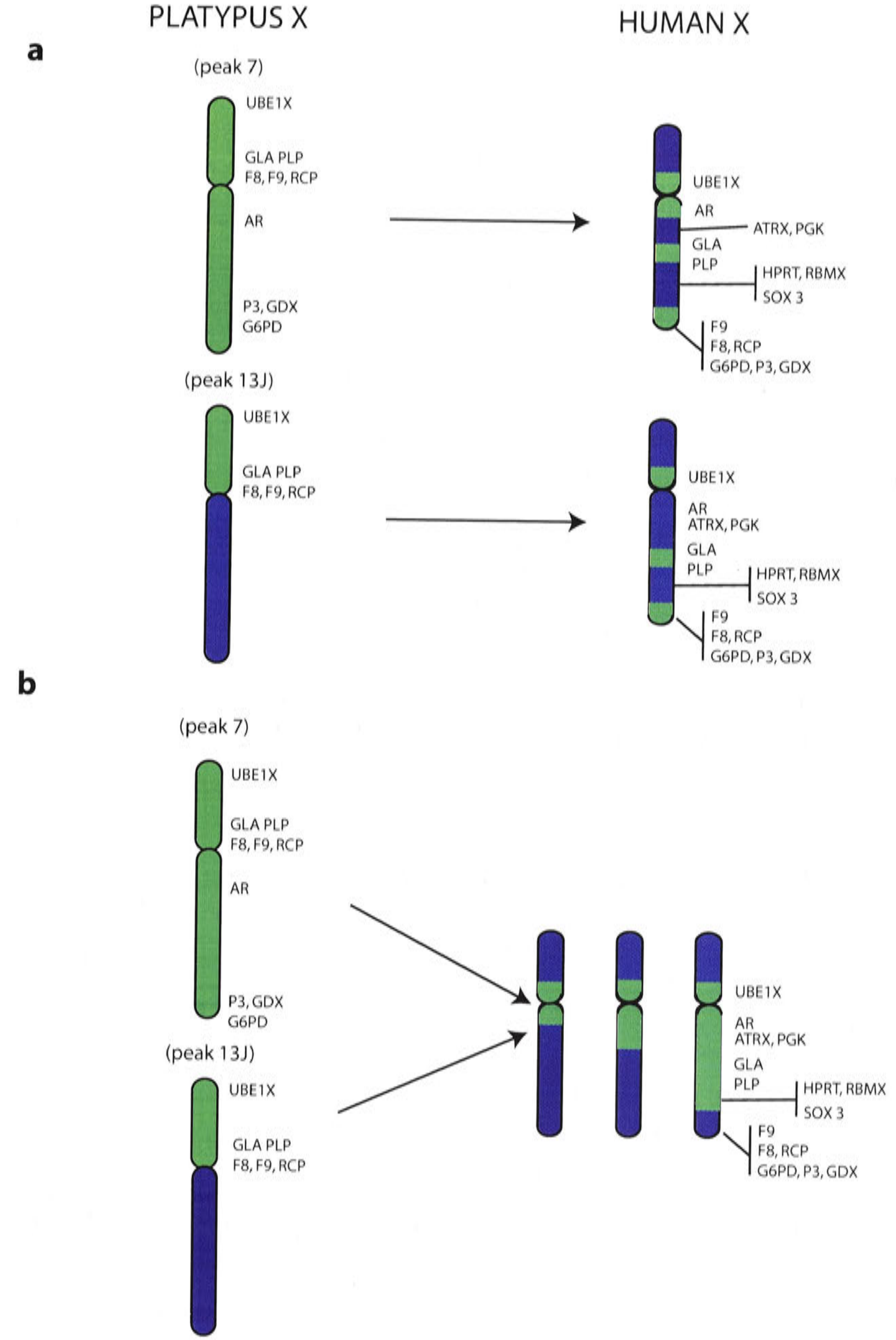


FIGURE 4.11: Sequences conserved between the platypus and human X. (a) The signal expected if hybridization were due to conserved unique sequences. (b) The observed graded pattern of signal (green) in different metaphase spreads was inconsistent within the pattern expected for conserved unique sequences.

Therefore, a signal should be observed above the centromere to Xp11.3, a band at Xq21.3 and at the terminal region of Xq from Xq26.3 to Xq28 (figure 4.11b). E2 should not hybridize to proximal Xq, since this region is autosomal in platypus.

The results obtained did not show these patterns. Firstly, no differential pattern was observed between the whole platypus X chromosome paint and the E2 paint. Secondly, the region containing *PGK, HPRT, ATRX, RBMX* and *SOX3*, all of which are absent from the platypus X, showed hybridization. Lastly, the terminal region of human Xq, which should have been hybridized with both paints, had little or no signal.

The whole X chromosome and the E2 paint hybridized to most of the Xq except for the terminus. This region of the human X chromosome is very gene rich, containing conserved genes such as *F8, F9, RCP, G6PD, P3* and *GDX* (figure 4.11b). These genes are found on the platypus Xp, and therefore the distal end of the human Xq (Xqter) should have hybridized with both the whole X chromosome paint and the E2 paint. These results seem to be inconsistent with the hypothesis that the conserved sequences are coding genes.

However, there are several possible explanations of why no signal was observed on human Xqter. The DOP-PCR may not have amplified this region of the platypus X chromosome. Alternatively, the gene positions in platypus, which relied on the older and less discriminant radioactive *in situ* hybridization with human cDNA probes, may be in error. If these genes map to autosomes rather than the X chromosome, the distal region of the human X chromosome is not part of the XCR but yet another independent addition to the therian X chromosome containing G6PD, *P3, RCP, F8, F9* and *GDX*. Other possible explanations include scrambling of the genes on the oldest part of the X to produce a scrambled signal over all of Xq.

More comparative mapping, using FISH with platypus probes, of genes from the conserved region of the X chromosome in platypus will better define XCR, XAR and the region present on therian but not the platypus X. In particular it will also confirm if the distal region of the X chromosome in eutherians really is part of the XCR.

4.3.3 Does painting reveal conserved X-specific inactivation "booster elements"

It has been proposed that there are "booster" elements, concentrated around the XIC and spreading out along the X chromosome, that transmit the signal that promotes and stabilizes X inactivation (Gartler and Riggs, 1983, Riggs et al., 1985). A specific

repetitive sequence (LINE – 1) was proposed by Mary Lyon to act as "booster" elements promoting X-inactivation (Lyon, 1998). LINE-1 elements are concentrated around *XIST* at human Xq13, and are more frequent in the ancient region of the X (XCR) than in the recently added XAR2.

The consistent hybridization of the human Xq13 region with the platypus X chromosome suggests that conserved repetitive sequences concentrated at the human XIC may also be found on the monotreme X. This could represent a conserved LINE repeat that is enriched in Xq13 and displays a graded signal over the rest of the human Xq reflecting a decreasing concentration away from *XIST*. Absence of signal from the human Xp implies that the added region has a low concentration of conserved repeats. This conserved repeated sequence is also found in paint derived from a small platypus autosome (peak 18) that hybridizes to a band in proximal human Xq, as well as to a small region of a human autosome pair (chromosome 16). If such a repetitive sequence is conserved on the X of monotremes, marsupials and eutherians, it may have an important conserved function in mammalian X-inactivation.

However, this idea is inconsistent with the idea that only young L1 (60-100 million years old) are enriched on the X chromosome (Bailey et al., 2000). This means either that a similar spread of L1 elements occurred independently in monotremes, or that old L1 elements were replaced by young ones in eutherians. After the divergence of Prototheria from Theria a subclass of L1 might have become active in Prototheria, retrotransposing throughout the X chromosome. As this L1 signal is enriched on the human X, it may belong to the L1M subclasses that can be found in most mammals and are enriched at human Xq13-21.

4.4 Summary

Painting of the flow-sorted platypus X to human Xq and proximal Xp showed that X chromosome sequences are conserved in all three extant mammalian groups.

The XCR is believed to represent an ancient mammalian X chromosome that is conserved in all three mammalian groups. The conserved sequence could be unique genes within the XCR shared by the X in all mammals, or conserved repeats that may be involved in X chromosome inactivation. The latter hypothesis is consistent with the observation that the strongest and most consistent signal observed was over the human Xq13-21 region, which contains XIST and has a concentration of L1 sequence.

The hybridization of the whole platypus X chromosome paint to most of the human Xq confirms gene mapping data for a conserved region, XCR, conserved between eutherians, marsupials and monotremes. However, the presence of strong signal at Xq13 does not support this hypothesis

Instead the results are more consistent with an early expansion of a LINE-1 as X-inactivation spread to keep pace with the degenerating Y chromosome. These LINE-1 sequences are thought to act as "booster" elements promoting and stabilizing X-inactivation.

Whichever theory is sustained, these experiments provide further evidence for the conservation of the mammalian X chromosome between eutherians and monotremes. This adds significance to the finding of a conserved chromosome position in mammalian sperm. It therefore seems that not only is gene content of the X chromosome conserved between the three mammalian groups, but also homologous chromosome position in sperm is conserved. Thus conservation of chromosome position may extend beyond the 50-60myr demonstrated for wombat and dunnart (chapter 3).

The conserved position of the sex chromosomes in sperm is specific to mammals, suggesting selection for a mammal important specific role after fertilization. One such pressure could be X-inactivation and dosage compensation. In marsupials X-inactivation is paternal, and this has been suggested to be the ancestral form (Cooper, 1971). The position of the X chromosome may induce paternal X-inactivation. This process may have evolved before the divergence of the three major mammalian groups, providing us with an explanation for the conserved X chromosome position observed in sperm. The next question that needs to be answered is how is the nuclear organization observed in sperm produced?

CHAPTER 5: CHROMOSOME ORGANIZATION DURING MEIOSIS

5.1 Introduction

During meiosis sperm develop through a series of morphological and cytological changes. These changes may play a key role in setting up chromosome organization and any epigenetic patterns important for zygote development.

At meiosis the pairing of autosomes occurs early in prophase, when homologous chromosomes come together and synapse at zygotene. Synapsis requires formation of a synaptonemal complex (SC) between the homologues, the main protein element of which is SCP3. Once the SC has formed, chromosomes start to condense and at pachytene crossing over occurs with the exchange of genetic material between the two parental genomes (Gilbert, 1997). The synaptonemal complex is vital for the pairing, recombination and segregation of homologues during meiosis.

As described in chapter 4, behaviour of the X and Y chromosomes differ from that of the autosomes at male meiosis in that there is little pairing, recombination only within the PARs, and the sex chromosomes become condensed and are preferentially located at the periphery of the nucleus to form the sex vesicle (SV) in its own separate domain. X-inactivation occurs in male pachytene cells, in a mechanism that includes *XIST* expression and mH2A localization to the SV. The association between the X and Y chromosomes disappears shortly after pachytene when the X and Y chromosomes migrate to separate poles at anaphase 1.

The X chromosome seems to be inactivated by pre-leptotene S-phase where the X chromosome becomes late replicating (Kofman-Alfaro and Chandley, 1970) and transcriptionally inactive as early as meiotic prophase (Monesi, 1965). The SV is essential for normal fertility as failure of the mammalian and *Drosophila* X and Y chromosomes to pair during these stages results in spermatogenic failure and arrest at prophase 1 or metaphase 1 (McKee et al., 1998). Similarly human patients and mutant mice, which are deleted for the PAR, are sterile (Mohandas et al., 1992).

Marsupial meiosis was extensively investigated in the 1920s because the small chromosome numbers of these species made observations easier, and was found to be very similar to that of eutherians (cited by (Setchell, 1977). However, there was no

pairing between the X and Y chromosome during meiotic prophase (Sharp, 1982). Also, sperm development was different in two ways. In marsupials, the acrosome is positioned asymmetrically on the dorsal face of the spermhead, and the tail is inserted in the mid-ventral surface of the head. All these features are due to the flattening of the nucleus, which in marsupials are 90° from the forming tail rather than parallel to the tail as in eutherians (Rodger, 1991).

In this study I used chromosome painting to observe chromosome organization in meiotic cells, synaptonemal complex antibodies to directly study the formation of the SC in marsupials, and electron microscopy to study chromosome organization and marsupial sperm development at a higher resolution.

5.1.1 Chromosome organization in mouse meiosis

Early radioactive *in situ* hybridization studies were able to determine the nuclear structure of Sertoli cells, which secrete testosterone and feed nutrients to the later spermatogenic stages. The nucleus of Sertoli cells contained one acidophilic body (the nucleolus) and two basophilic bodies that radioactive *in situ* hybridization with mouse satellite DNA proved to be chromocentres. This technique also provided evidence that chromocentres lie at the centre of the nucleus in round spermatids (Pardue and Gall, 1970).

There is surprisingly little literature on the use of chromosome paints to study chromosome organization at meiosis. However, one study used paints derived from mouse chromosome 5, 11, 13, 15, 16, 17, X and Y to observe chromosome organization in mouse meiotic cells. In spermatogonia all chromosomes showed distinct non-overlapping spatial domains as in somatic nuclei, with chromosomes 5, 11, 15 and 17 confined towards the periphery of the nucleus, whereas chromosomes 13 and 16 had a more internal position. The X and Y chromosomes were positioned far apart at the periphery of the nucleus (Garanga et al., 2001). In pachytene cells each autosome pair maintained a distinct position within the cell nucleus. In Sertoli cells the Y chromosome was preferentially confined near one of the two chromocentres.

In robertsonian fusions one of the chromosomes paired with the homologue involved in the translocation and remained in the same position observed in normal mouse spermatocytes. However, the other chromosome involved in the translocation did not pair with its homologue and the homologue had an abnormal position within the

spermatocyte. The end result is pairing of one of the homologues but a lack of synapsis and recombination between the other homologue involved in the translocation. In these animals, very few cells are produced by the end of meiosis II. Therefore, correct nuclear architecture at prophase 1 is essential for synapsis, recombination and segregation of meiotic chromosomes (Garanga et al., 2001). In this study we will use chromosome paints to determine if chromosomes have non-random positions in marsupial meiotic cells, which may be essential for proper segregation and development of sperm.

5.1.2 The SV

A SV can be found at the periphery of pachytene cells in male eutherians (Metzler-Guillemain et al., 2000) and marsupials (Sharp, 1982). The SV is characterized by its condensation, transcriptional inactivation and lack of recombination. Suppressing recombination between the mammalian sex chromosomes is important as to not break up the male-specific package of SRY and spermatogenesis genes found on the Y. The condensation of the SV aids in rendering it inaccessible to transcriptases and recombinases. The SV is characterized by specific proteins and expression of the *XIST* gene, which work together forming a stable inactive chromatin body (Ayoub et al., 1997).

The properties of the SV differ between animals. The human SV and the chimpanzee SV are extremely condensed, whereas the mouse SV is relatively decondensed (Metzler-Guillemain et al., 2000). The differential condensation of the SV could be due to the amount of heterochromatin in chimpanzees and humans.

The shape and the peripheral position of the SV are due to attachments of the chromosomes to the inner surface of the nuclear envelope (Metzler-Guillemain et al., 2000) (figure 5.1). The human X chromosome bends at the centromere at prophase 1. The two telomeres at either end of the X chromosome associate with each other, bending the X into a horse shoe shape, within which the Y chromosome is found.

Four different SV systems have been described in mammals. The X-Y chromosomes may synapse and recombine within a small PAR, as in mouse and man (Solari, 1980). Alternatively, full X-Y parasynapsis can occur over the entire length of the chromosomes, which undergo, however, no recombination or exchange of genetic information (eg. soft-furred field rats, (Nanda and Raman, 1981). In some species, such as the sand rat, the X and Y chromosomes are asynaptic but remain associated end to

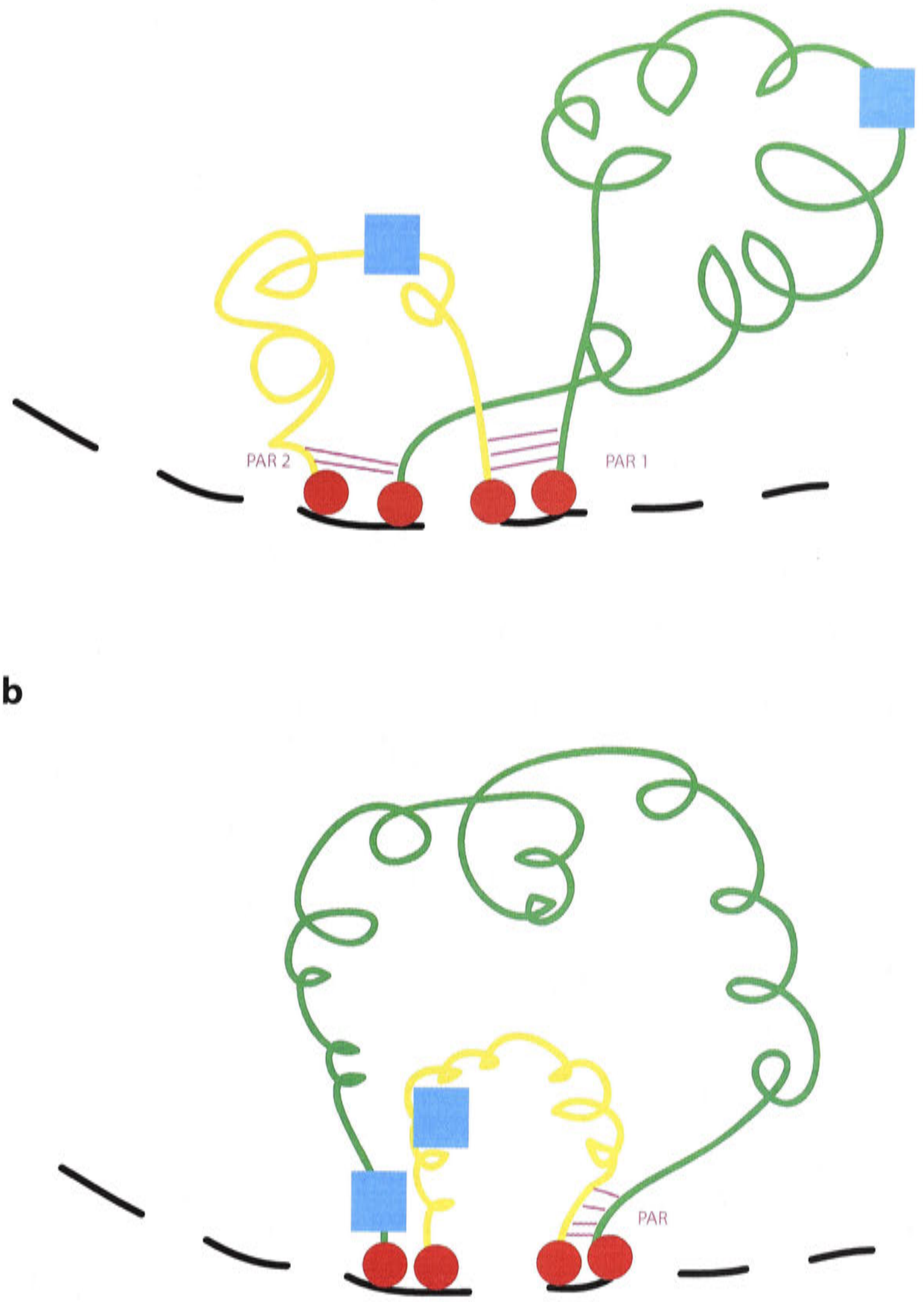


Figure 5.1: Sex vesicle organization in human (a) and mouse (b). Green represents the X chromosome and yellow the Y chromosome. Black lines represent the nuclear membrane, ■ the centromeres, ● the telomeres.
Modified from Metzler-Guillemain, 2000.

end (Solari and Ashley, 1977), and in marsupials, the X and Y chromosomes show no end association or physical contact (Sharp, 1982).

5.1.3 SV proteins

Some proteins, unique to the SV are involved in regulating condensation and inactivation of the SV. The murine HP-1 like chromodomain protein (M31) spreads along the whole XY body from mid pachytene and covers the SV by late pachytene (Motzkus et al., 1999), where heterochromatinization and transcriptional inactivation of the X chromosome occurs (McKee et al., 1998). The mouse SV condenses only when M31 localizes to it at mid pachytene (Motzkus et al., 1999), suggesting that M31 plays an important role in heterochromatinzation.

Other proteins involved in the condensation and inactivation of the SV include XY40, XYbp, XY77, p51 and mH2A. XY40 is a SV specific protein that is confined to the axial elements of the sex chromosomes (Smith and Benavente, 1992). As yet its function is unknown, though it may play a role in SV structure and nuclear organization. The XYbp gene is ubiquitously expressed as a 4.2Kb transcript, and also has a 2.8Kb testis specific transcript. It encodes a protein with a ring-finger domain. Ring finger domains participate in protein-protein interactions and are associated with processes in gametogenesis (Parraga and del Mazo, 2000). Monoclonal antibody to the XYbp protein localized with the X and Y chromosomes in spermatocytes. This protein also localizes to centrosomes and seems to be an important part of the centrosome structure (Parraga and del Mazo, 2000). XY77 localizes to the entire XY body in late pachytene cells but is not found in early to mid stage pachytene cells (Kralewski et al., 1997). The function of this protein is not yet known, but since it is not present in early stage pachytene cells, it is unlikely to play a role in forming the SV. Instead it may maintain the condensed state of the SV, which is at its greatest in later pachytene cells. Protein p51 is distributed over the XY body but also localizes to centromeric heterochromatin of autosomes in germline cells, where it may be involved in the heterochromatinization (Smith and Benavente, 1992). All of these proteins work in conjunction to produce the condensation observed in the SV, and may also play an active role in inhibiting recombination.

A histone variant, mH2A, is also found on the SV and will be discussed in detail in chapter 6.

5.1.4 Marsupial SV

Little is known about the SV of marsupials. It appears to differ slightly from that of eutherians, because there is no synaptonemal complex between the X and Y chromosome. The SV of marsupials is based on a dense plate between the ends of the sex chromosomes (Solari and Bianchi, 1975); Sharp, 1982).

In the marsupial *Thylamys elegans*, SCP3 antibody hybridized to the dense plate in mid pachytene cells. The dense plate, which is an extension of the axial elements of the sex chromosomes, include proteins that are phosphorylated indicating that the dense plate is subjected to specific regulation processes (Page et al., 2002). The axial elements of the sex chromosomes always made contact at one or both ends, and the contact may be between both short arms, both long arms or by the X chromosome long arm and the Y chromosome short arm.

After the autosomes have paired, the telomeres of the X and Y chromosome attach to the nuclear membrane and the dense plate is formed. Marsupial species with autosomal translocations on their sex chromosomes (*Potorous tridactylus* and *Macrotis lagotis*) do have a SC between the translocated autosome and its pair, but this does not extend to the sex chromosomes (Sharp, 1982).

In the 1980's it was suggested that eutherian sex chromosomes might pair by associations of large amounts of heterochromatin (Wolf et al., 1988). However, the X and Y chromosomes of some macropodid species (eg. *Macropus eugenii* and *M. rufogriseus*) share heterochromatic regions, but show no evidence of pairing (Toder et al., 1997, Sharp, 1982).

5.1.5 Aim of this study

In this study I have used chromosome painting, protein immunohistochemistry, and electron microscopy to look at the relationship of the X and Y chromosome at meiosis in three marsupials, the slender mouse opossum (*Marmosops incanus*), the tammar wallaby (*Macropus eugenii*) and the fat-tailed dunnart (*Sminthopsis crassicaudata*). Chromosome painting was used to observe if the chromosome arrangement was non-random in marsupial pachytene cells and to compare with the pachytene nuclear organization in chicken. Chromosome painting was used to look at the SV in a South American marsupial, *Marmosops incanus* (2n=14) and also in the tammar wallaby. SCP3 antibody was used to examine the synaptonemal complex in the

fat tailed dunnart as a comparison with the results of Sharp *et al,* 1982. Electron microscopy was used to look at the relationship of the X and Y chromosomes at higher resolution. Sperm of some rodents and the dunnart have different regions of condensation (Breed, 1993, Breed, 1997, Soon et al., 1997). In the dunnart sperm this region contains DNA packaged with histones (Soon et al., 1997). I therefore wanted to determine if any of these regions of DNA condensation occurred in tammar wallaby sperm.

5.2 Results

5.2.1 Organization of chromosomes in marsupial meiosis

5.2.1.1 Attempts to prepare tammar meiotic stages

In mouse the meiotic cycle is well known. Twelve different kinds of tubules, each with different stages occurs in mouse seminiferous tubules. For instance, in a stage 1 seminiferous tubule is found spermatogonia A, pachytene cells, stage 1 spermatids and stage 13 spermatids (Oakberg, 1956). This makes it possible to select the stage of interest. However, the stages of development of the tammar wallaby testis are not as well known and not enough samples are available to develop a method to observe all stages of development in the tammar wallaby. Such a method would require multiple samples from different animals at different age groups.

Electron microscopy studies have demonstrated 14 steps of spermiogenesis (Lin and Jones, 2000), but the timetable of meiotic cell development in the marsupial testis is unknown. This made it extremely hard to obtain the different meiotic stages required for this study. The most common cell types observed were spermatogonia, pachytene cells, round spermatids and spermatozoa. Spherical Spermatogonia and spermatids were of little use as they lack reference points in which to orientate chromosomes, something essential for 2-D analysis when not using such techniques as erosion analysis (Croft et al, 1999). Primary spermatocytes were more useful as they allowed the study of the tammar wallaby SV structure, which could also be used as a reference point in looking at chromosome organization. Unfortunately few metaphase or diakenetic stages were obtained, but pachytene stages were observed.

5.2.1.2 Chromosome organization in pachytene cells

Tammar testis was pulled apart with tweezers and a scalpel, placed in a hypotonic, fixed with methanol: acetic acid and visualized with a 2-D microscope. This resulted in the destruction of the three dimensional structure of the pachytene cells and

severely limited the observations on chromosome organization in tammar wallaby pachytene cells.

2-dimensional analysis was used to count a large sample size of tammar pachytene cells hybridized with probes derived from chromosome Y and 7 (figure 5.2a). The relationship of chromosomes 6 and 7 were also studied, though with a much smaller sample size (figure 5.2b). The results obtained for chromosome Y and chromosome 7 showed no consistent relationship between the two chromosomes. 30 pachytene cells were studied with the two chromosomes in close association in 11 cells (37%), on opposite sides of the nucleus in 9 cells (30%), and with one chromosomes at the periphery and the other chromosomes towards the interior of the nucleus in 10 cells (33%). This suggests random positioning of chromosomes in pachytene cells, but the 2-dimensional analysis and fixative techniques do not allow an accurate representation of the chromosome position within the cell. This will be discussed in detail later in the chapter.

Chicken testis underwent the same preparation technique as the tammar wallaby. Through 2-dimensional microscopy it was impossible to interpret chromosome position in relation to each other though a small sample size was used and a larger sample size is needed to produce accurate results. There did seem to be a radial distribution of the macrochromosomes but this may have been an artefact of the fixation technique, which collapses the cell (figure 5.3).

5.2.2 Sex chromosomes and the marsupial sex vesicle

5.2.2.1 Sex chromosome relationship in the tammar wallaby SV

The organization of the SV was investigated by chromosome painting in the tammar wallaby (2n=16). In this species there is a heterochromatic region shared between the long arm of the Y chromosome (Yq) and the short arm of the X chromosome (Xp). Chromosome painting was used to determine if the shared heterochromatic region paired in the SV through double painting the heterochromatic region with the X and Y probes. If two separate heterochromatic regions were visualized with chromosome painting, it meant heterochromatic regions were not pairing, but if only one heterochromatic signal was consistently observed, it would mean they are in close association and may be pairing. Paints used were derived from a

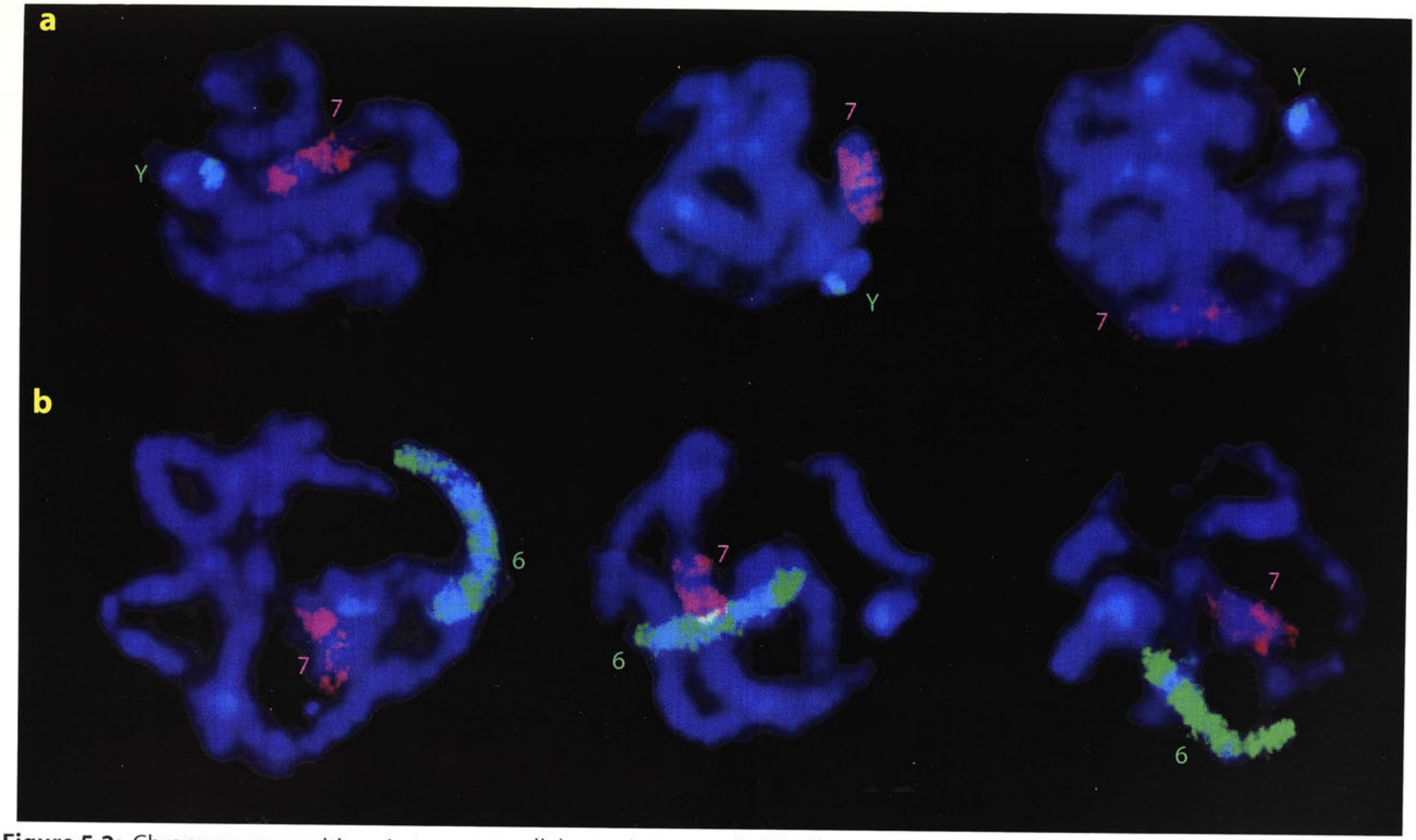


Figure 5.2: Chromosome positions in tammar wallaby pachytene cells. (a) Chromosomes Y (green) and & 7 (red) showed no consistrent association. (b) Chromosomes 6 (green) and 7 (red) also showed varying degrees of association, though 2-dimensional microscopy made this hard to determine.

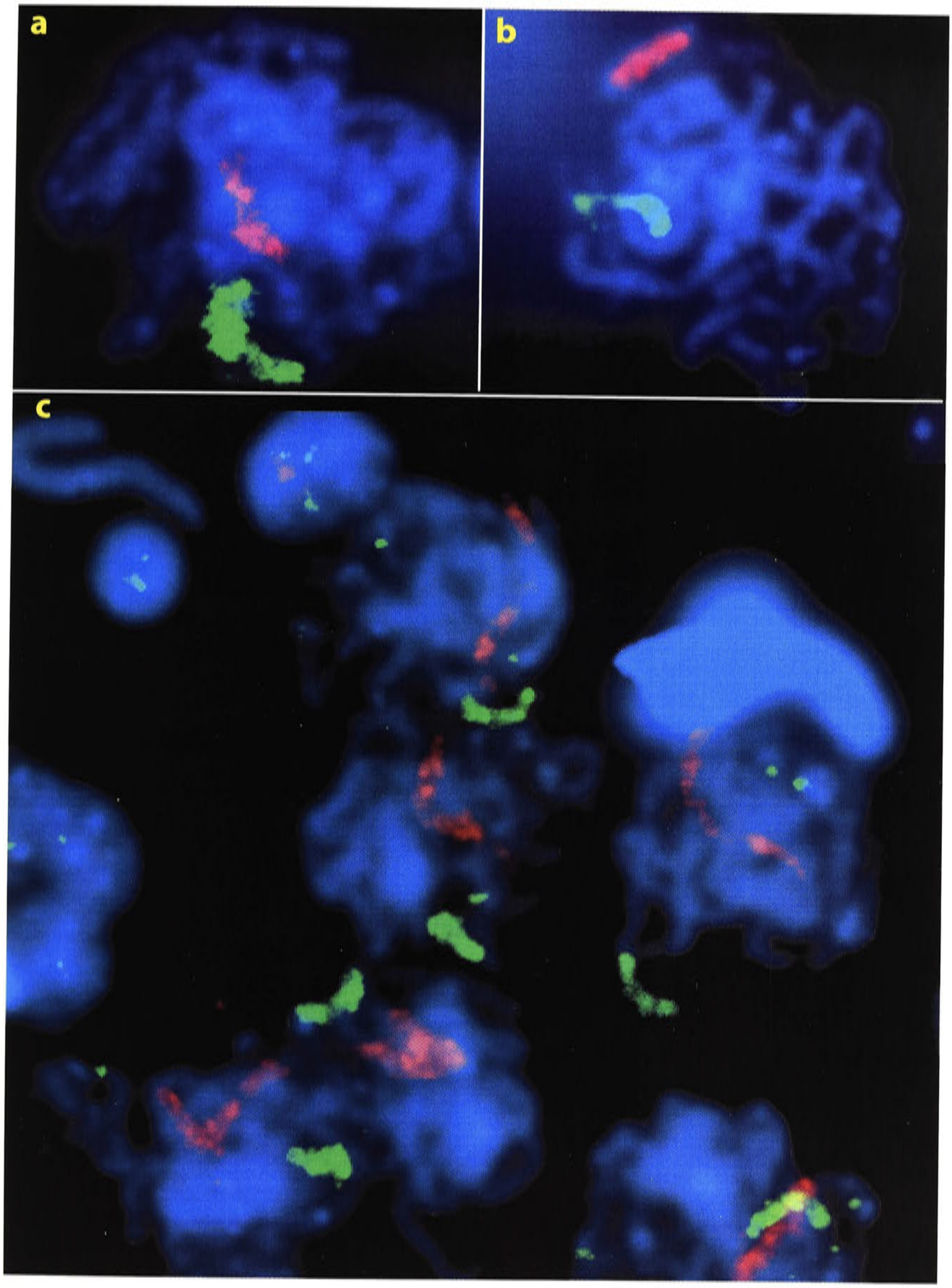


Figure 5.3: Chromosome position in chicken pachytene cells. (a) Chromosomes 8 (green) and Z (red). (b) Chromosomes Z (green) and 9 (red). (c) Field of six pachytene cells, with two spermatids and one spermatozoa. Chromosome 9 (green) and chromosome 2 (red). No clear chromosome associations were observed, but the macrochromosomes did seem to have a radial position, although this may be an artefact of the fixation technique.

microdissected Xq and Y (which is too small to sort, Toder et al., 1997). The Xq paint enabled the position of the X chromosome to be determined within the sex vesicle.

The X chromosome was not condensed at the zygotene, but became condensed to form an SV at pachytene (figure 5.4a). The SV seemed to be evenly divided between the Xq and Y chromosome paints as expected, since Xp and the Y chromosomes both stain with the Y paint. There was little merging of the chromosome paints suggesting that the two signals were not closely apposed, as they would be if they paired (figure 5.4b-d).

The whole X chromosome paint was then used along with the Y paint to observe if the heterochromatic regions paired. In these images the long arm of the X chromosome is seen in green and the Y chromosome is seen in red. As both the X paint (green) and the Y paint (red) hybridized to the heterochromatic regions, a yellow signal would indicate the hybridization of both paints to the heterochromatic regions on Xp and Yq. If only one region of co-localization between the two paints were observed (yellow region) it would indicate pairing of the X and Y chromosomes at the heterochromatic shared sequence. However, two separate co-localized signals would indicate that the heterochromatic regions were in different areas of the SV and therefore were not paired.

The pattern of the heterochromatic sequences differed between pachytene cells. In some pachytene cells, the two heterochromatic regions were on separate sides of the sex vesicle, indicating that they do not pair (figure 5.5a), whereas in other pachytene cells the heterochromatic regions were in close association indicating either pairing or viewing in two different planes (figure 5.5b). The donut shape of the sex vesicle in some pachytene cells indicates end-to-end association (figure 5.5c), with the heterochromatic regions not in close association (figure 5.5c).

5.2.2.2 Sex chromosome relationship in the slender mouse opossum

Preparations of *Marmosops incanus* (a South American marsupial) meiotic cells were supplied by Dr. Marta Svartman. The prepared slides contained most phases of meiosis, which is a rarity in marsupial meiotic preparations. The *M. incanus* X chromosome was flow-sorted by the Malcolm Ferguson-Smith laboratory, Cambridge University, UK. The *M. incanus* X chromosome was painted onto meiotic preparations in order to observe the relationship of the X and Y chromosome through different stages of meiosis.

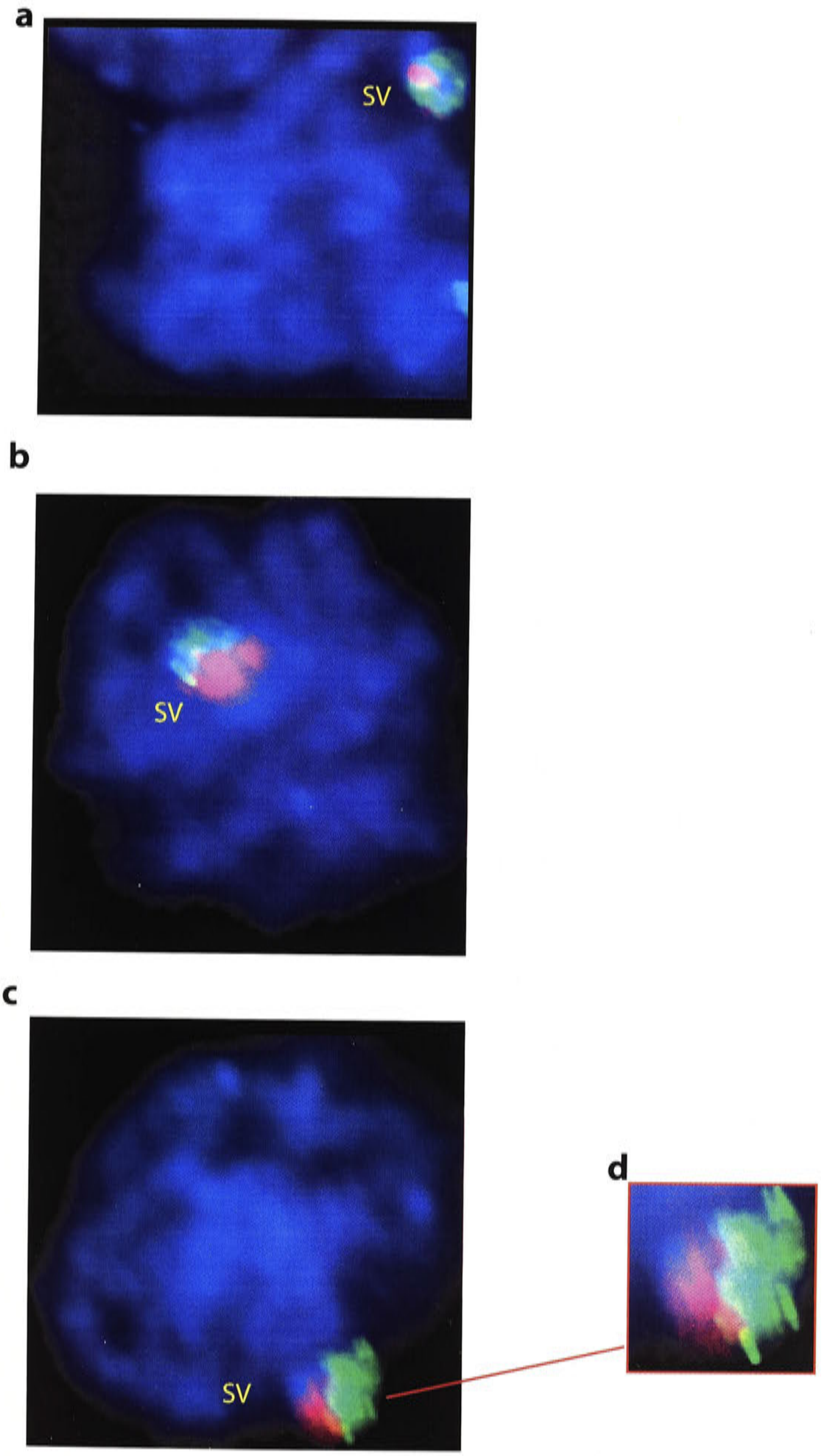


Figure 5.4: Meiosis in the tammar wallaby with Xq (green) and the Y chromosome (red). a-b) Sex vesicle has formed in pachtyene cells. c) Chromosomes kept together in a balloon shape with close associations. d) Higher magnification of the sex vesicle, showing little or no pairing between Xq and the Y chromosome, which would have appeared yellow.

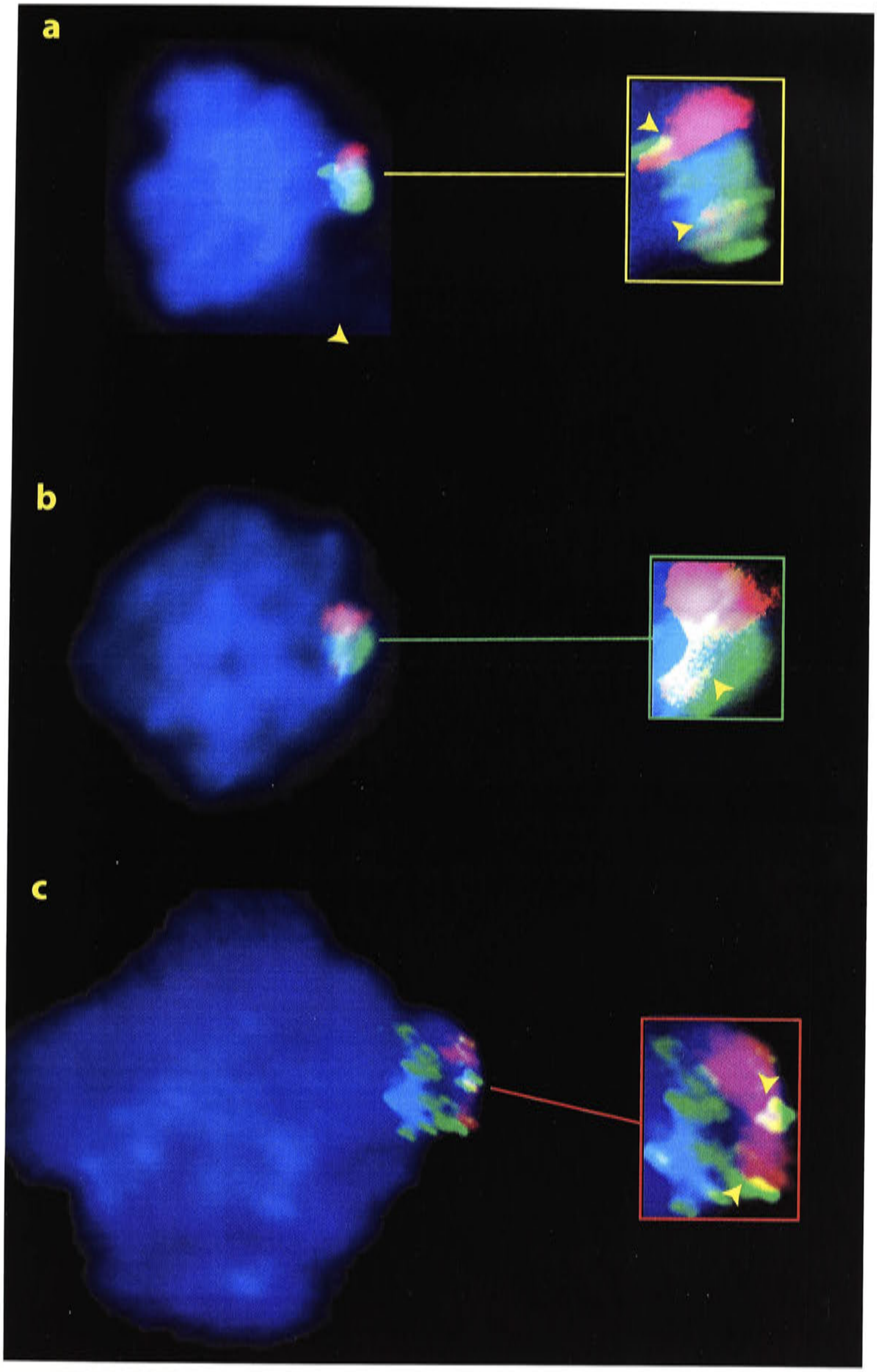


Figure 5.5: Tammar wallaby pachytene cells painted with X (green) and Y (red). Yellow regions indicate co-hybridization of both paints, indicating the shared heterochromatic region. (a) Yellow regions separate indicate no pairing (➤). (b) One large yellow region indicates that the heterochromatin may be paired. (c) Telomeric end to end association between the X and Y chromosomes. The different yellow regions again suggest no pairing between the heterochromatic regions.

In leptotene cells the X chromosome had not yet formed the sex vesicle (figure 5.6a). At zygotene, the chromosomes had begun to condense and the X chromosome lay at the periphery of the cell nucleus (figure 5.6b). In pachytene cells the X chromosome assumed a U shape, with the ends of the X chromosome associating with the ends of the Y chromosome (figure 5.6c). In most pachytene cells the SV (containing the X chromosome and Y chromosome) had a balloon shape (figure 5.6d). In diakenesis there were numerous chiasmata between autosomal homologues and the X and Y chromosomes still seemed to be in close proximity to each other (figure 5.6e). At metaphase I the X chromosome and Y chromosome were no longer paired (figure 5.6f).

Due to the quality of the autosomal paints, autosome arrangement in *M. incanus* meiosis was not studied.

5.2.2.3 The Synaptonemal complex in the dunnart

Chromosome painting demonstrated no evidence of chromosome pairing between the X and Y chromosomes, but to confirm this result, a synaptonemal complex antibody (SCP3) was used to observe if a synaptonemal complex, and therefore pairing, occurred in the dunnart. The synaptonemal complex protein 3 (SCP3) forms part of the lateral element, and it is extremely conserved, so the antibody against the mouse protein reveals the SC in marsupials.

One problem encounted was the clumping of antibody to areas where DNA had degraded. The method used for making the preparations appeared to cause damage to the nuclei of some cells, and these damaged areas were highlighted by the antibody sometimes obscuring any SC signal. Although the SV could be seen in some cells, in other cells it was impossible to determine where the SV was located in the absence of any intense DAPI stain.

The beginning of synaptonemal complex formation occurred in early pachytene cells. More than 20 short SC regions were observed over the six autosome pairs, implying that the formation of the synaptonemal complex begins at multiple sites along the chromosomes (figure 5.7a). No synaptonemal complex formation was seen within the heterochromatic SV seen by DAPI staining (figure 5.7a). At later stages of formation longer SCs were representing the pairing of the homologous chromosomes. More than six signals were observed in which some homologues were completely paired, and other homologues were still in the process of pairing. The fork observed in these cells represented the pairing of the two homologues taking place (figure 5.7b)

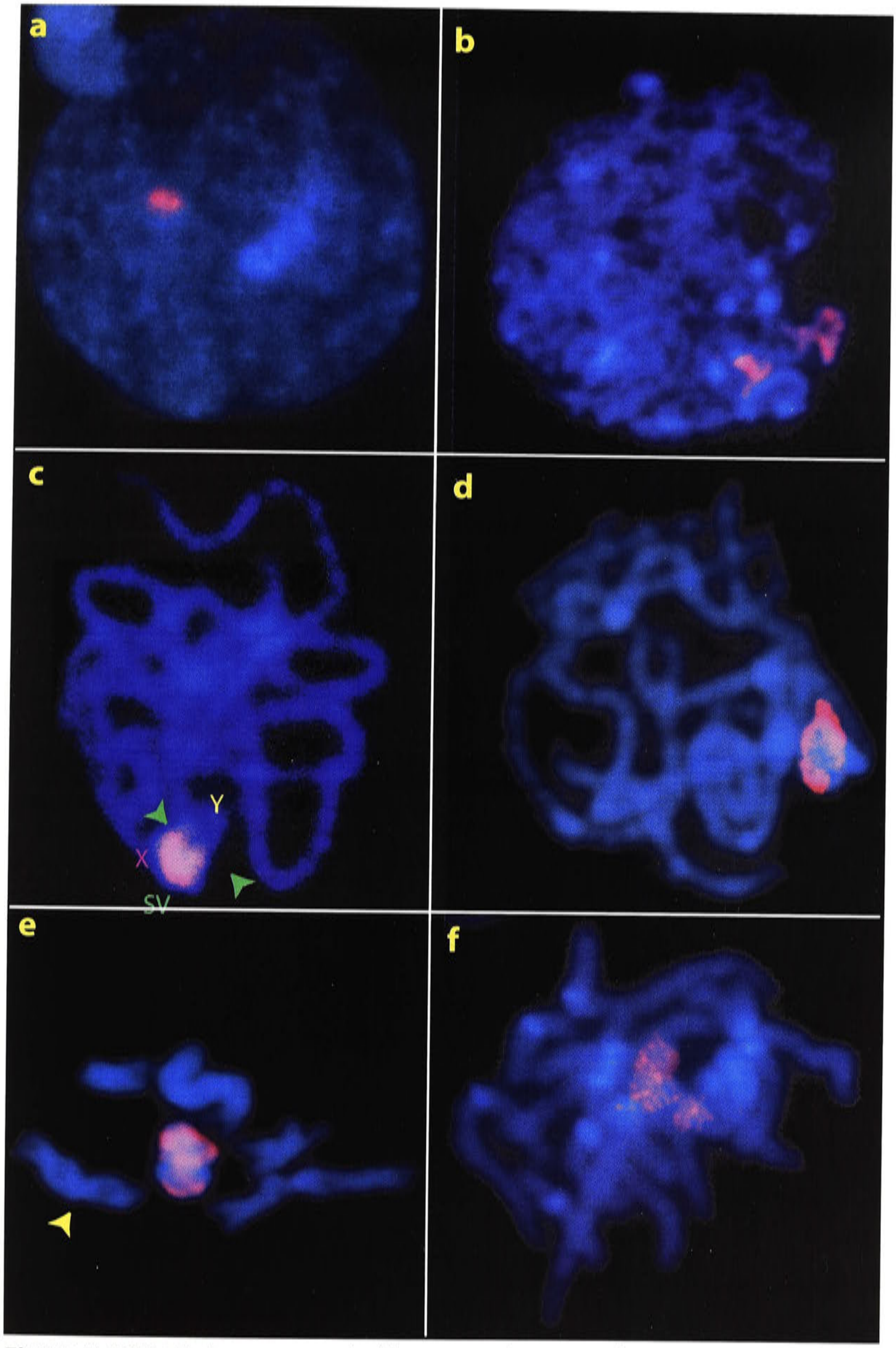


Figure 5.6: The X chromosome in *Marmosops incanus* primary spermatocytes was visualized with chromosome painting. (a) Leptotene cell . (b) Zygotene cell. (c-d) Pachytene cells, showing telomeric associations with the X and Y. The Y chromosome can be recognized as the part of the SV with no hybridization (➤). (e) Diakensis, showing crossing over events and the X chromosome (➤). (f) The X chromosome in a metaphase cell.

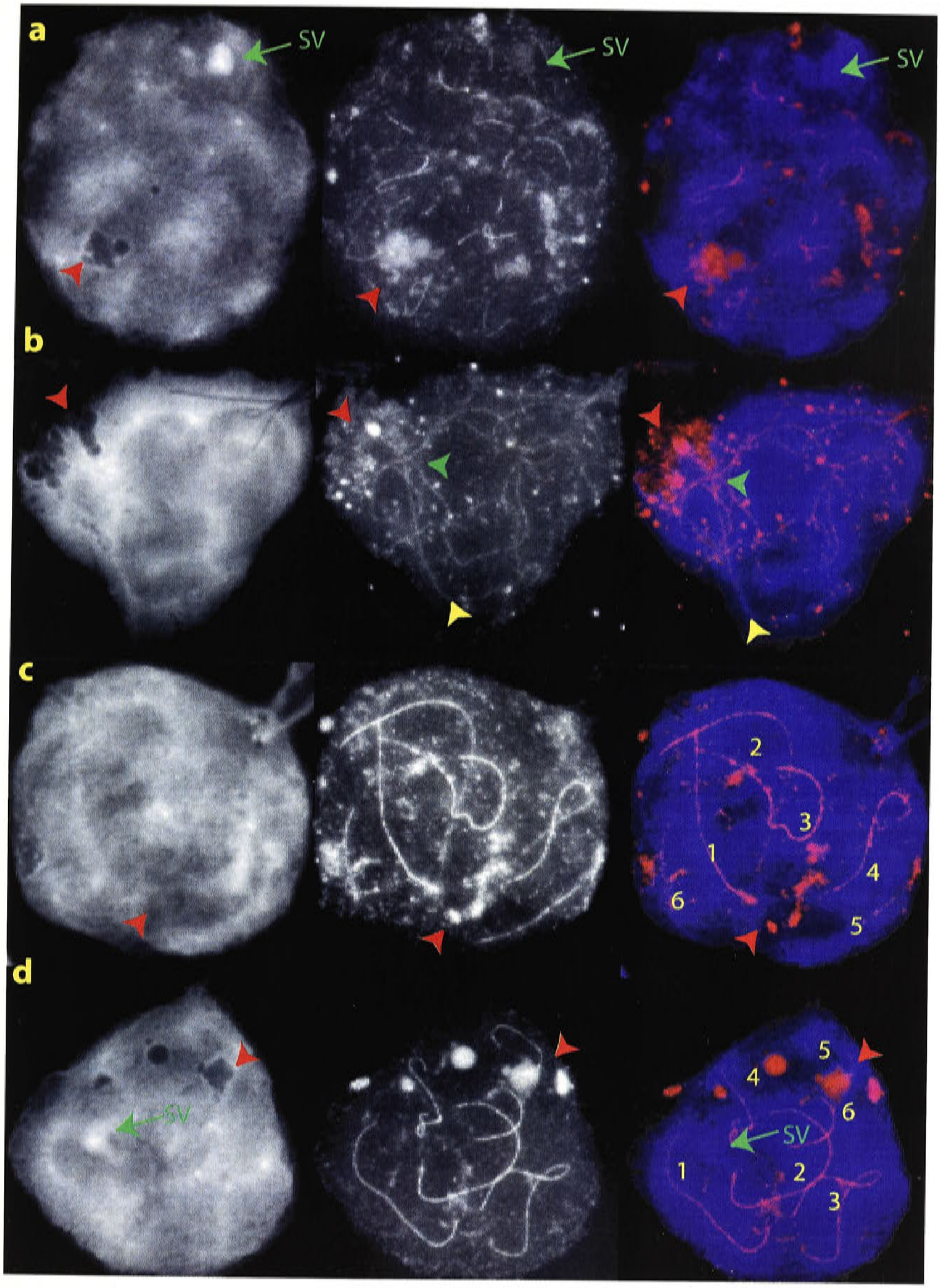


Figure 5.7: Synaptonemal complex formation in dunnart pachytene cells visualized by SCP3 antibody. (a) Early on the synaptonemal complex (SC) begins at multiple points. (b) Next stage shows pairing of different homologues at different stages. Some are already paired (➤) and others are in the process of pairing (➤). (c) SC has completely developed over six autosomes, but not over the sex vesicle. (d) bright DAPI staining corresponds to the sex vesicle at the pachytene stage. Antibody clumps at regions where DNA degradation has occurred (➤).

In cells in which SC formation was completed, only six SC were ever observed, each one representing an autosomal pair (figure 5.7c). In these cells, the X and Y chromosome within the SV lacked an SC (figure 5.7d), corroborating the results previously obtained by Sharp in the dunnart. This is consistent with my conclusions from 5.2.1 that the X and Y chromosome do not undergo homologous pairing in marsupial male meiosis.

5.2.3 Electron microscope observations of meiosis and spermatogenesis in the tammar wallaby

Transverse cross-sections through primary spermatocytes allowed closer observation of the SV and cell organization within the seminiferous tubule. Scanning electron microscopy of freeze fracture preparations allows observations of the cell organization within the seminiferous tubule. Freeze fracturing involves fixing the seminiferous tubules, freezing them in liquid nitrogen, and then breaking them. Transmission electron microscopy permits a much higher magnification of cells, allowing features to be accurately observed that cannot be interpreted with light microscopy. In this study I particularly wanted to observe the relationship of the X and Y chromosome in the SV and the different developmental stages of marsupial spermiogenesis.

5.2.3.1 Scanning electron microscope study of tammar wallaby meiosis

Mrs. Lily Cheng from the Electron Microscopy Unit RSBS, ANU, generated some scanning electron microscope images of tammar wallaby meiotic cells, which were useful in showing their overall organization within a seminiferous tubule. Large round cells, representing spermatogonia and primary spermatocytes were seen at the outer edge of the seminiferous tubule, and a tangled mess of spermatozoa lay towards the centre of the seminiferous tubule (figure 5.8a).

The SEM pictures show stages in the development of the spermhead. The acrosome forms a scoop shape on the dorsal surface of the immature spermhead (figure 5.8b and 5.9a) after which the midpiece and tail are attached. The ventral surface of the sperm has no characteristic features, but the asymmetric shape of the spermatozoa is obvious (figure 5.9b).

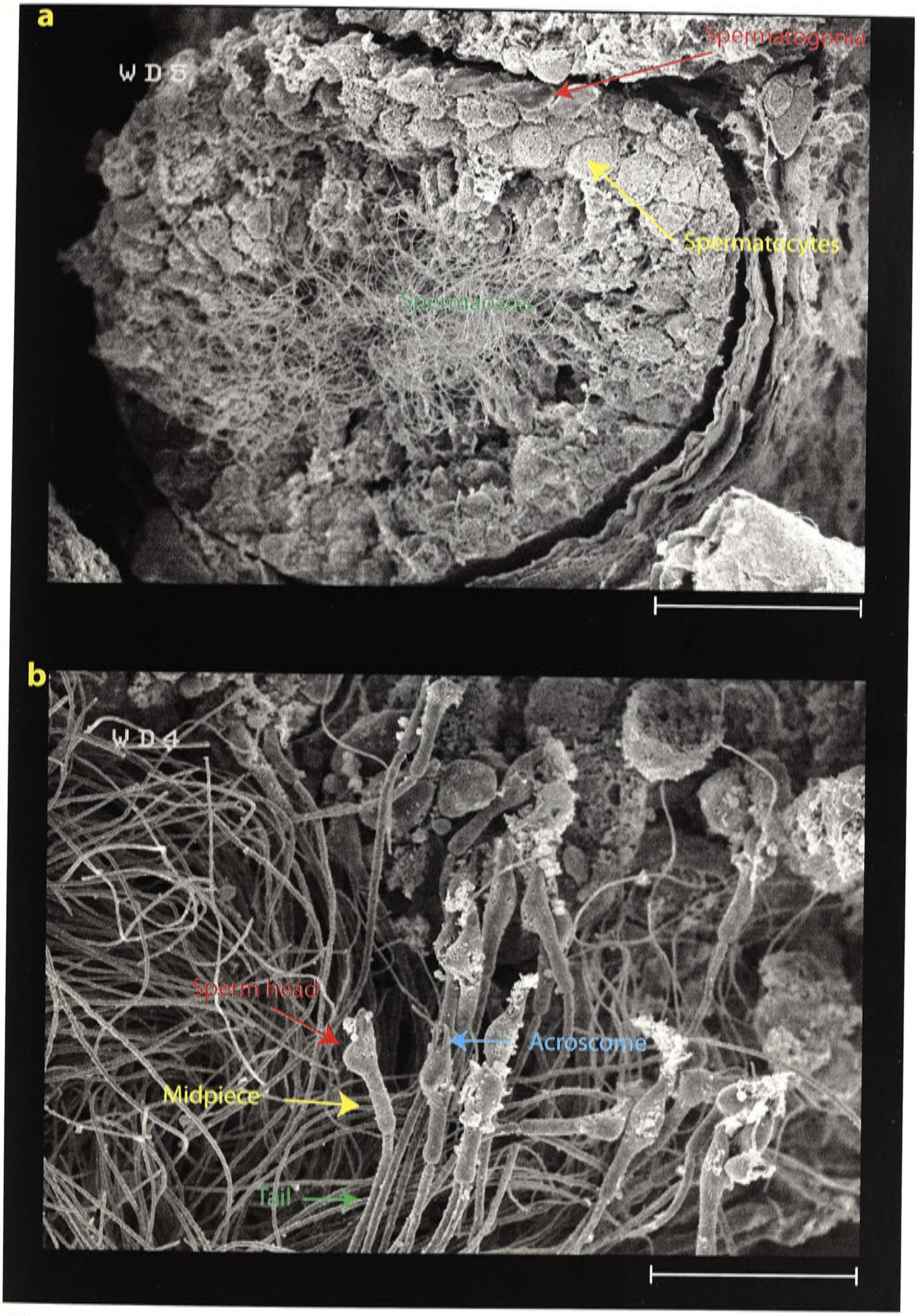


Figure 5.8: SEM of tammar wallaby testis. (a) Seminiferous tubule, showing cell type changes from the outside to the centre of the tubule. (b) Immature sperm, with a scoop shaped acrosomal cap. (a) bar = 66.7µm; (b) bar = 20µm.

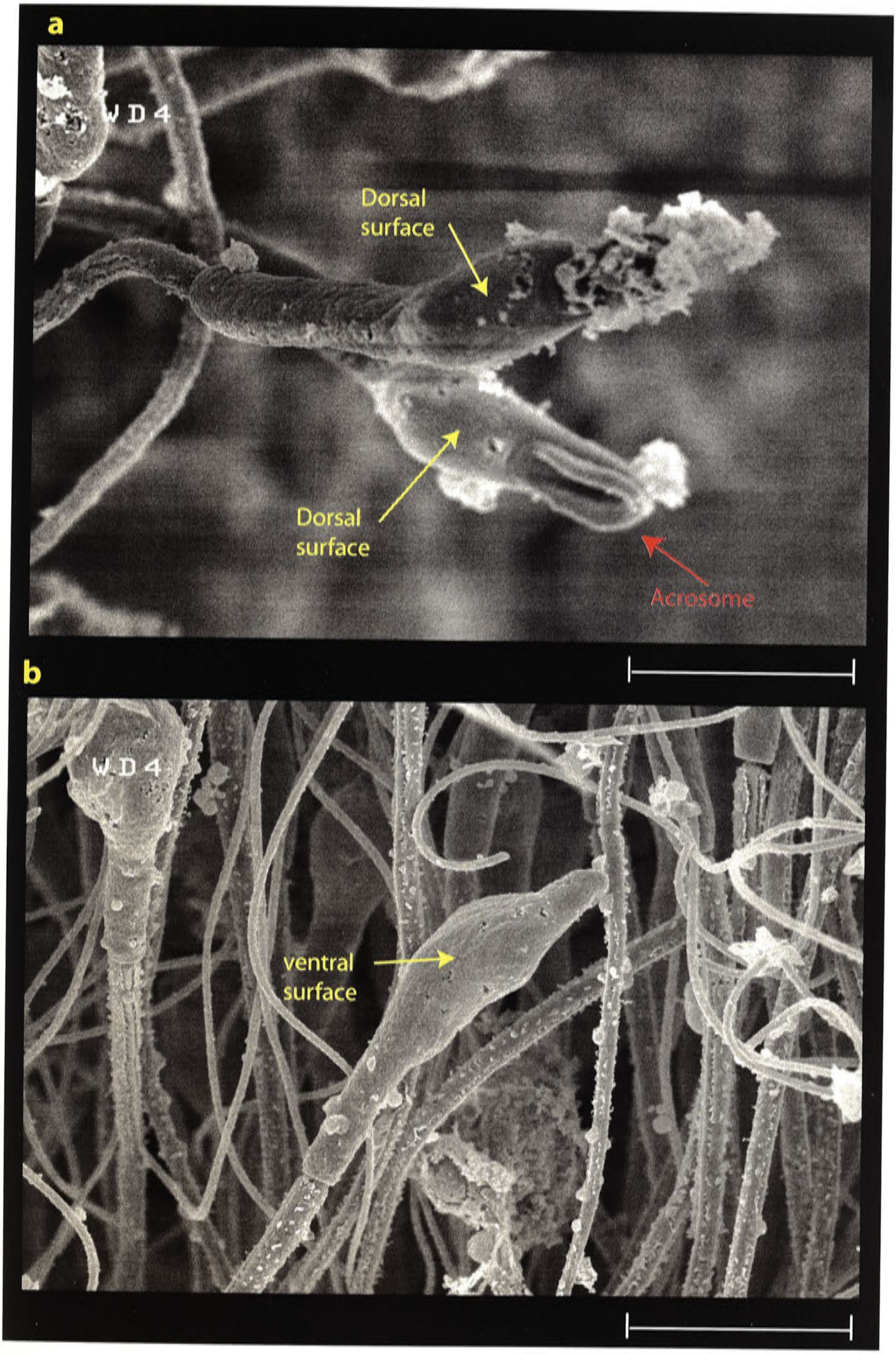


Figure 5.9: SEM of immature tammar wallaby sperm. (a) Dorsal view of an immature sperm. (b) Ventral view of an immature sperm. (a) bar = 5µm; (b) bar = 6.67µm

5.2.3.2 Transmission electron microscope study of the tammar wallaby

Numerous images were taken of different stages of meiosis in the tammar wallaby and used to examine different cell types at different stages of spermatogenesis.

Only a few of the many testis cell types could be distinguished and their characteristics were examined. In Sertoli cells, which feed nutrients to the meiotic cells, the nucleolar organizing region (NOR), was identified from published images of tammar testis in which condensed regions of DNA are adjacent to the NOR (Lin and Jones, 2000) (figure 5.10a and b). Condensed DNA is dark in staining allowing chromosomes to be identified with an electron microscope picture.

Several pachytene cells with condensed chromatin were examined by transmission electron microscopy (figure 5.11a). Each contained a condensed region of DNA which could be interpreted either, as the NOR, or the SV (figure 5.11a). In one pachytene cell, the condensed region formed a horseshoe shape around a small less condensed region of DNA (figure 5.11b), which resembled the SV observed using chromosome painting. Therefore, this condensed region of DNA was interpreted as the SV rather than the NOR. The outer horseshoe shaped region could represent the X chromosome whereas the inner region represents the Y chromosome. Higher magnification of this region revealed, little or no pairing between the two regions, supporting the chromosome painting observations that showed no pairing between the X and Y chromosomes in marsupial SVs and the absence of synaptonemal complex (figure 5.11b).

Spermatogenic stages were simple to interpret as the developing sperm nuclei were condensed and heavily stained (figure 5.12a). With electron microscopy it was possible to study the progression of chromatin condensation and sperm development.

The sperm nucleus began to condense in round spermatids, the haploid products of meiosis. At this stage the acrosome is beginning to form, but the tail has not yet been attached (figure 5.12b). In a sagittal section the spermhead is observed to have just started to take its asymmetrical shape. The connecting piece has yet to be attached to the spermhead (figure 5.12c). In later stages of spermatogenesis the tail has begun to form and is connected to the spermhead and the acrosome lies on the dorsal side of the transverse sectioned spermhead (figure 5.13a). At these stages more lightly stained regions of DNA can be observed, that represent less condensed regions of DNA. It is possible that these regions contain histone packaging that is passed through to the

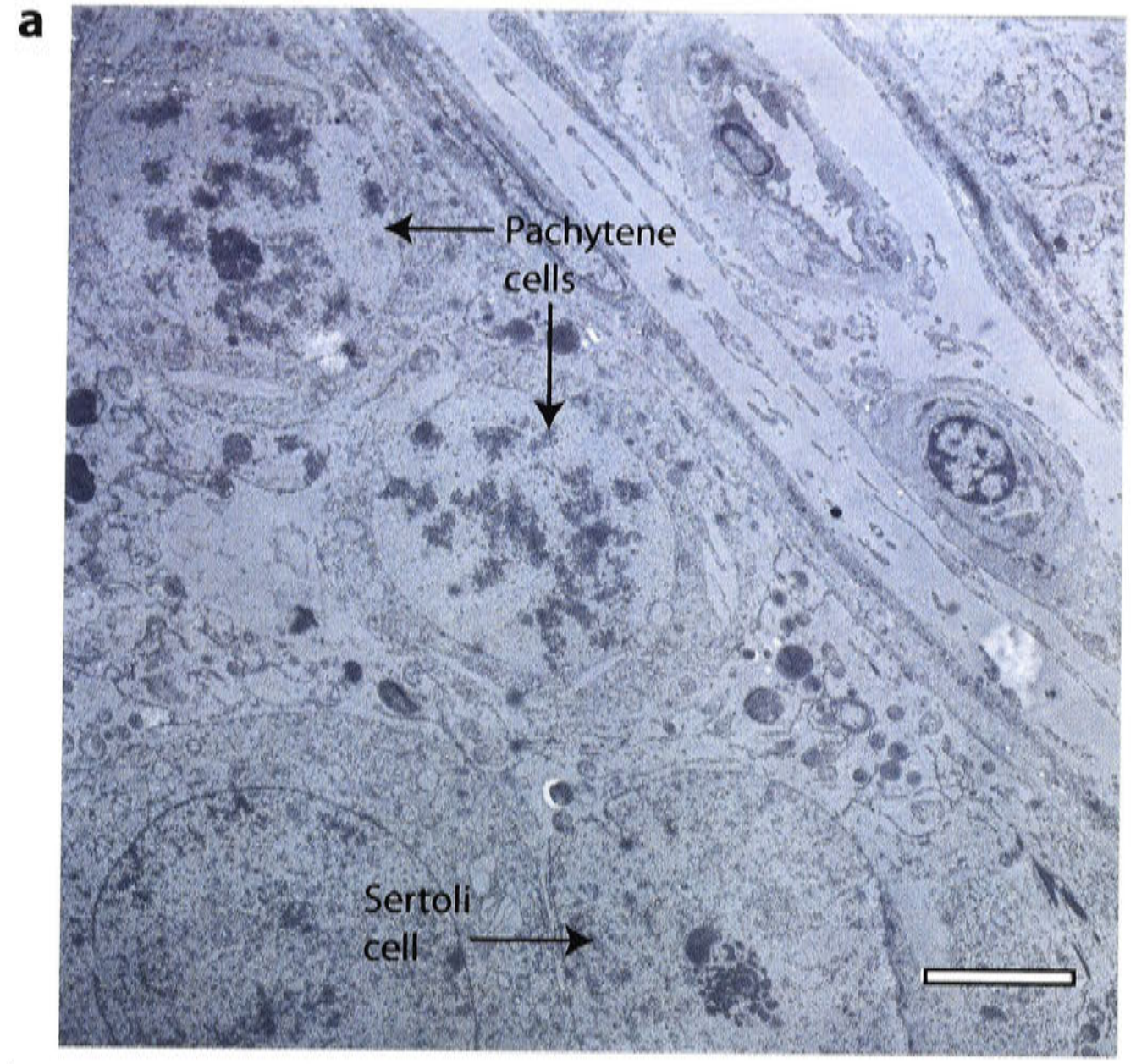
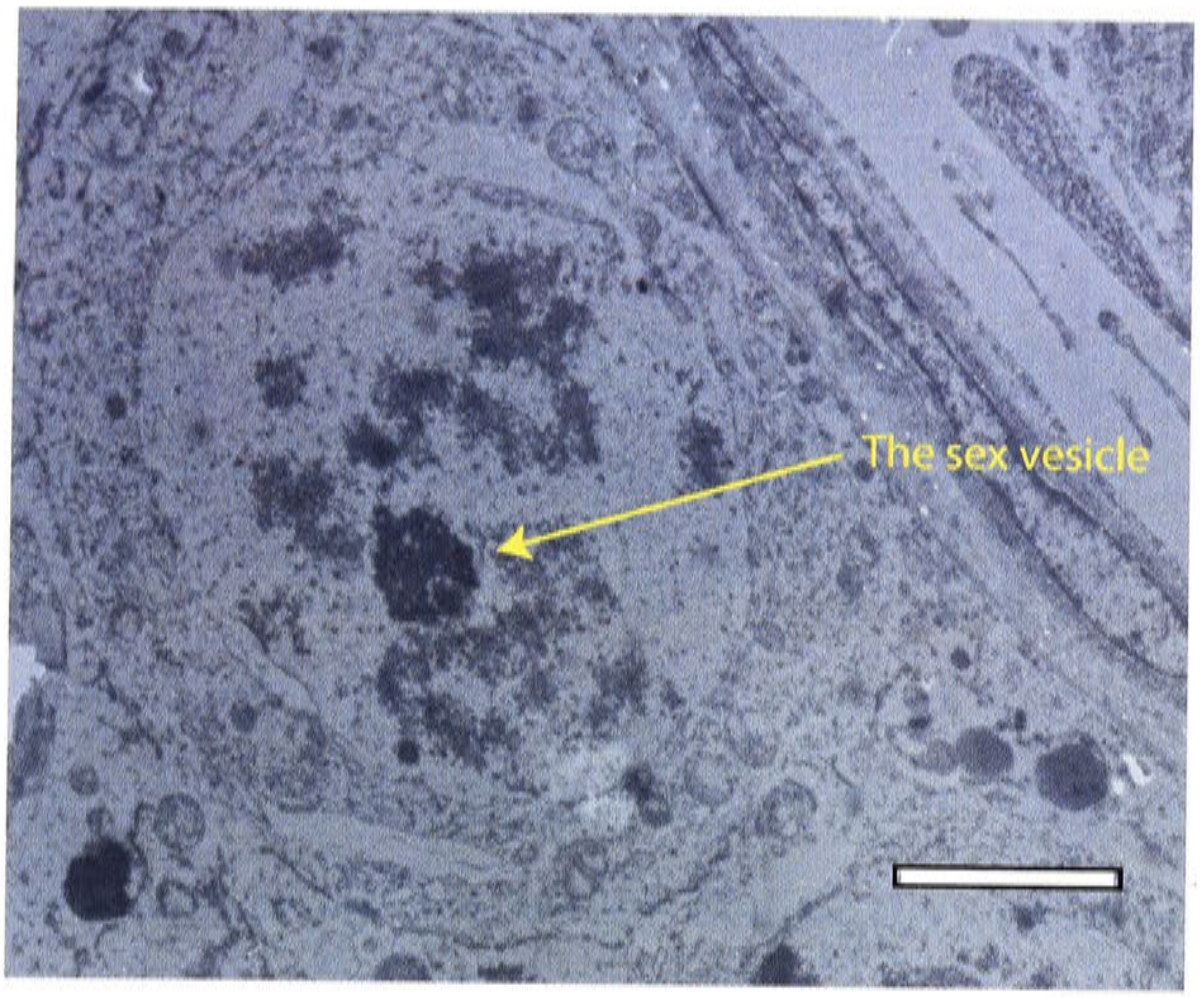


Figure 5.10: Tammar wallaby meiotic cells. (a) 2 pachytene cells and a Sertoli nuclei (b) Sertoli cells are characterized by a large nucleolar region and an adjacent region of condensed DNA, which is a chromocentre (Krzanowska *et al.*, 2000). (a)bar = 5.0µm, (b) bar = 1.6µm.

a



b

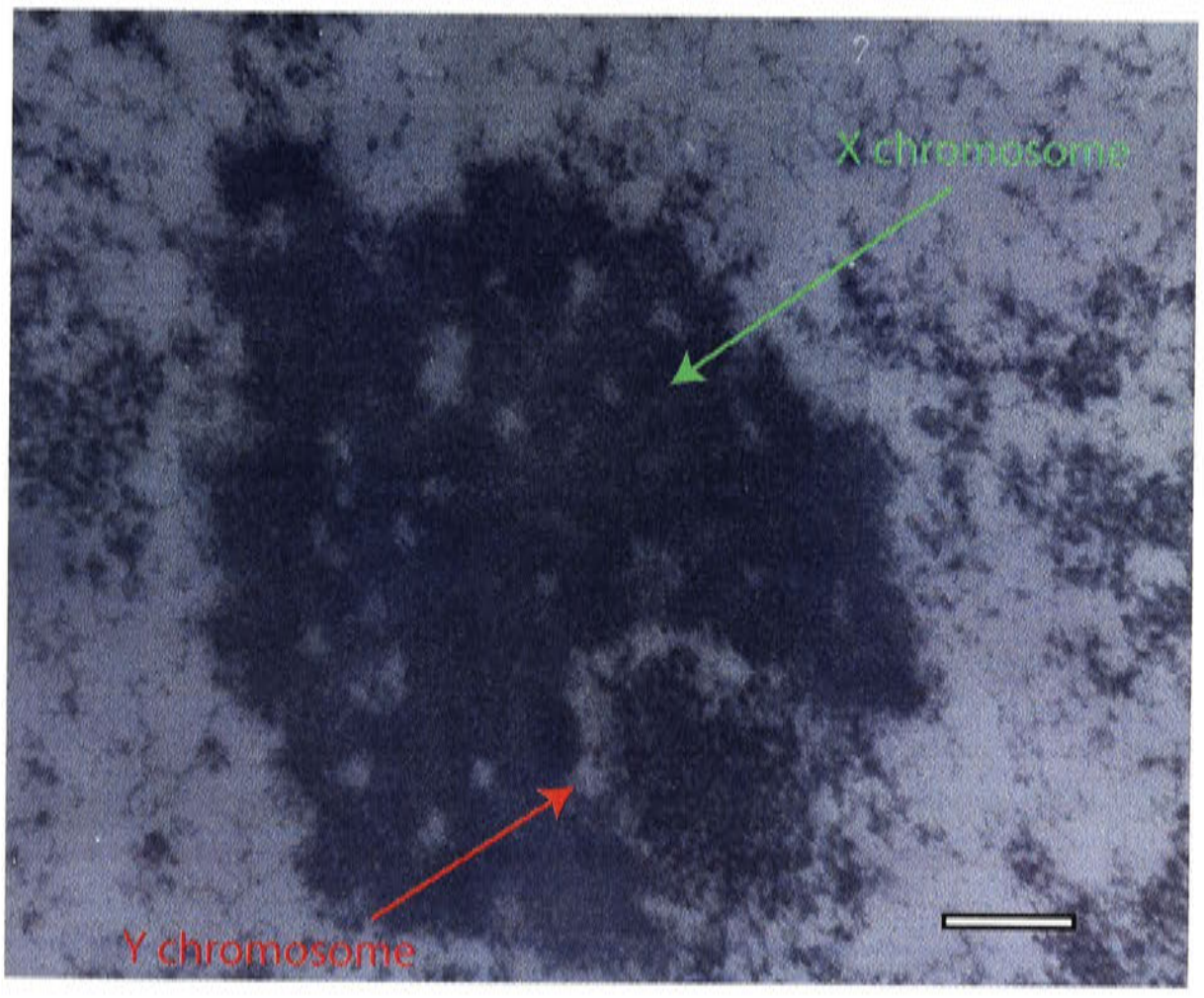


Figure 5.11: SV in a tammar wallaby pachytene cell nucleus. (a) Balloon shaped condensed SV region. (b) The condensed outer region may represent the X chromosome, and the region within this the Y chromosome. No pairing is observed between the X and Y chromosomes.
(a) bar = 3.3µm, (b) bar = 250nm.

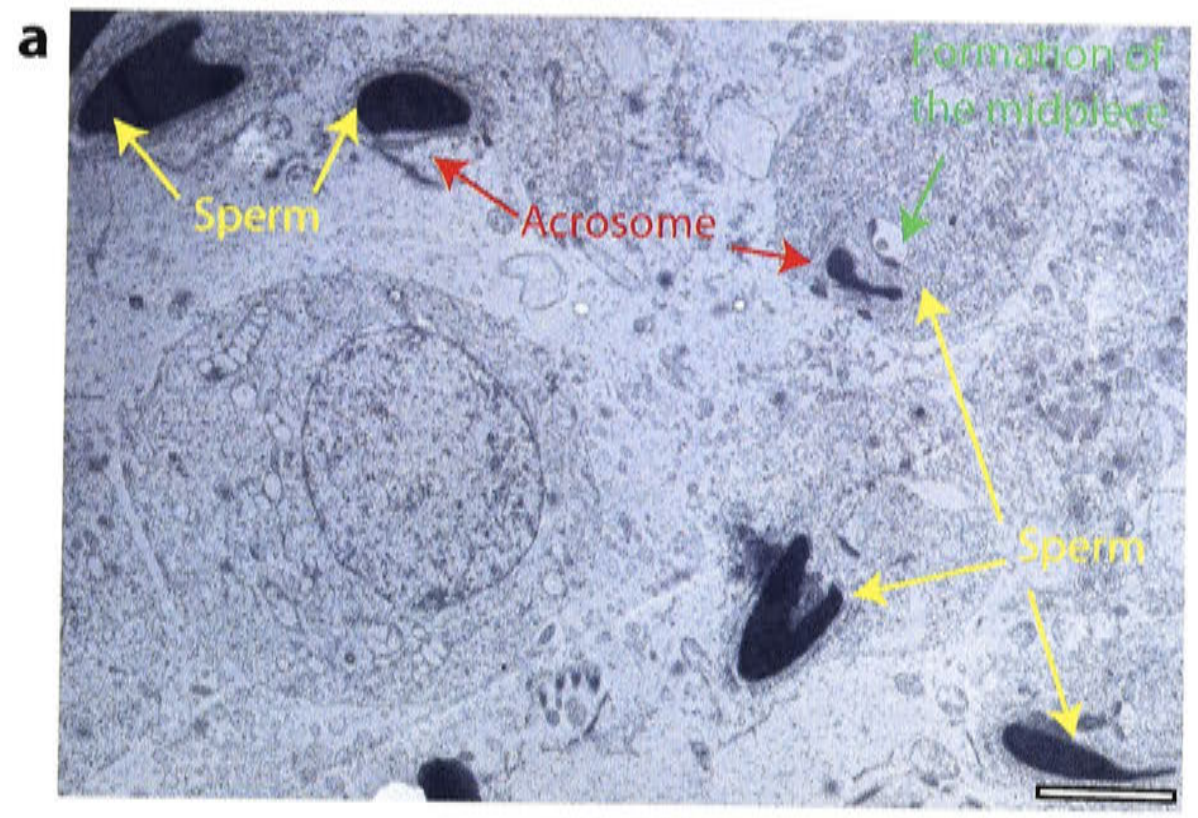
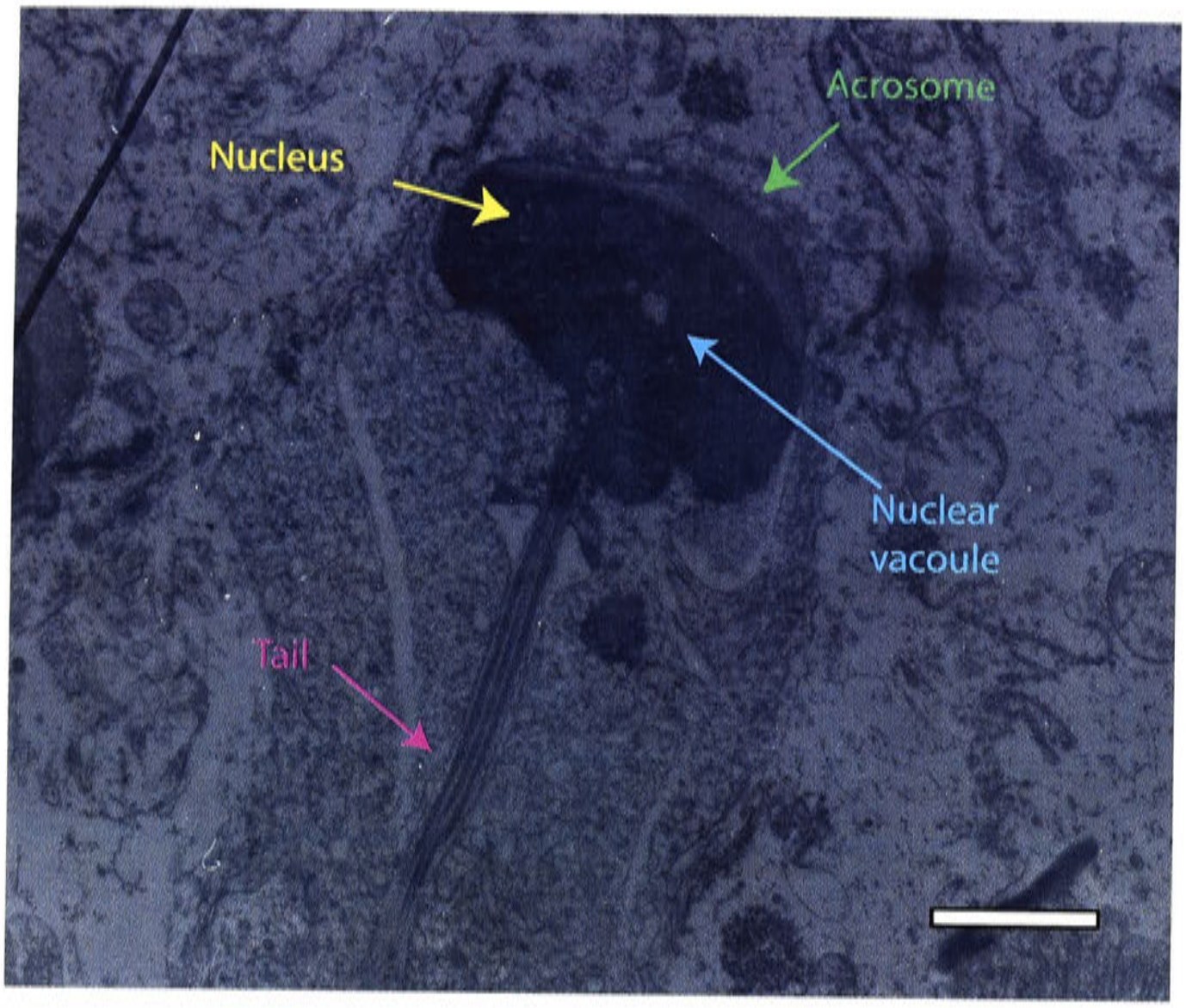


Figure 5.12: Tammar wallaby spermatogenesis. (a) Tammar wallaby spermatogenesis. (b) Round spermatid. (c) Elongating spermatid.
(a) bar = 5.0µm; (b) bar = 833nm; (c) bar = 833nm.

a



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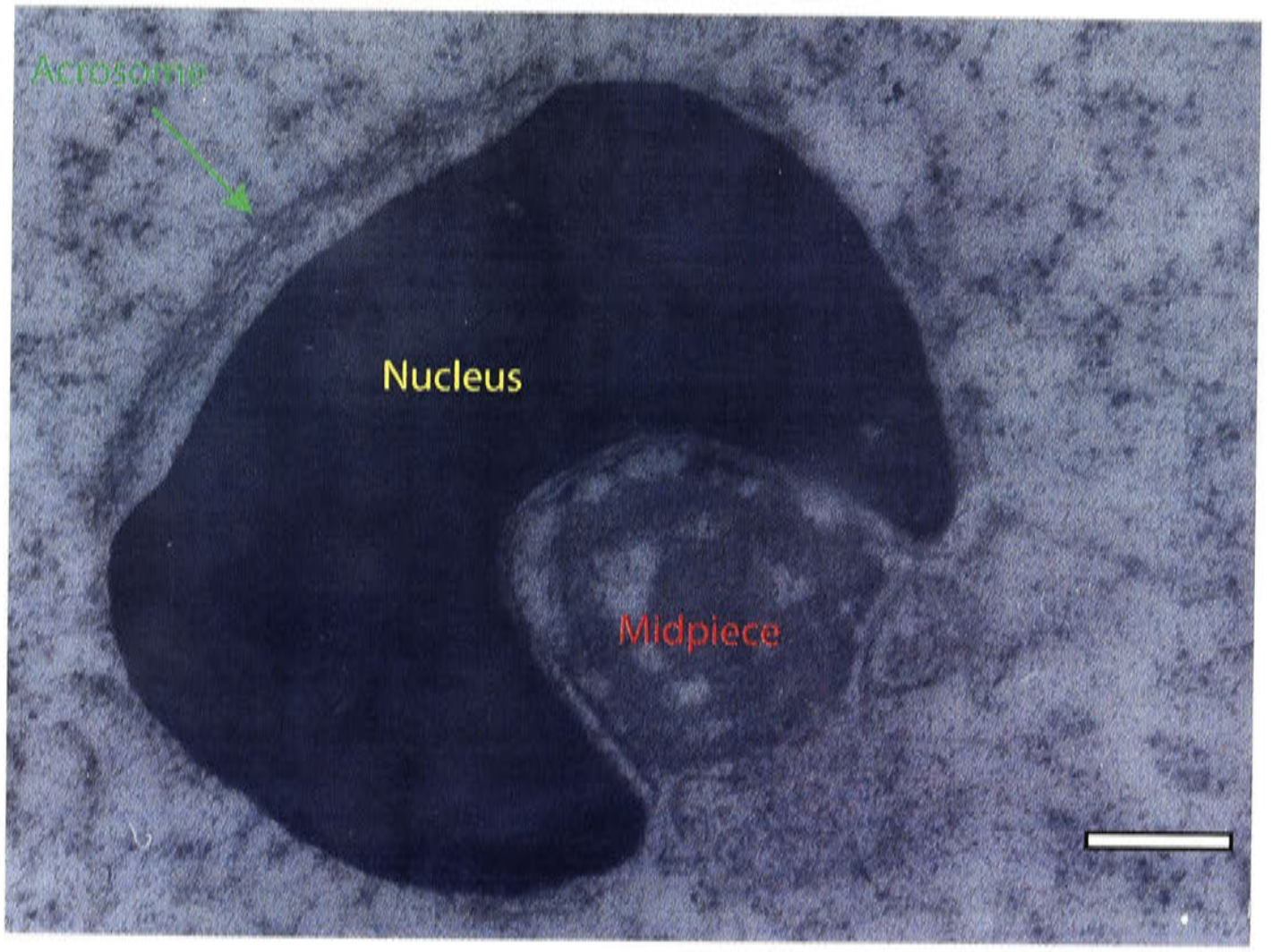


Figure 5.13: Transverse sections of tammar wallaby sperm. (a) Beginning of the formation of the tail. Nuclear vacuoles can be seen in the nucleus. (b) Transverse section of a sperm nucleus, showing the dorsal position of the acroscome. (a) bar = 1.4µm, (b) bar = 333nm.

zygote. Previous work using chromosome painting suggested that chromosomes assume an arch shape in the sperm nuclei (Greaves et al., 2001). A TEM transverse section illustrates how the arched spermhead morphology would impose this shape in the chromosomes (figure 5.13b).

5.3 Discussion

5.3.1 Chromosome organization in tammar wallaby pachytene cells

The goal of this study was to understand nuclear organization in marsupial meiosis and to see how this related to the highly structured chromosome arrangement in sperm (chapter 3). I was unable to compare the tammar X and Y chromosomes in all stages of meiosis and spermatogenesis, as diakenesis, metaphase 1 and 2, anaphase 1 and 2, were never observed in the tammar wallaby.

A detailed observation on the chromosome organization of the tammar Y and 7 in pachytene cells was undertaken. This showed that the association of the chromosomes was highly variable. However, these results may have been affected by 2-dimensional microscopy and the fixation techniques used.

2-dimensional microscopy involves looking down on the cell from above and therefore, it is impossible to see the depth of the nucleus and where a chromosome fits into the cell. If the chromosomes are on opposite ends of the cell, but both signals are facing straight up, it would seem as if they are in close association (figure 5.14a). 2-dimensional analysis can be accurate if the two chromosomes are in close association on the side of the membrane (figure 5.14b).

The chromosome preparations were placed in a hypotonic and fixed with methanol: acetic acid. This fixative is good for fixing DNA, but it is not good at keeping the 3-dimensional structure (Kozubek et al., 2000). This fixative collapses the cell nucleus, which is not a problem in marsupial sperm due to the flattened morphology, but is a problem in pachytene cells that are spherical. 2-dimensional analysis also makes it incredibly difficult to understand the three dimensional nature of the cell, as chromosomes that are on opposite ends of the cell nucleus, may seem to be in close association due to the collapsing of the cell nucleus (figure 5.14c).

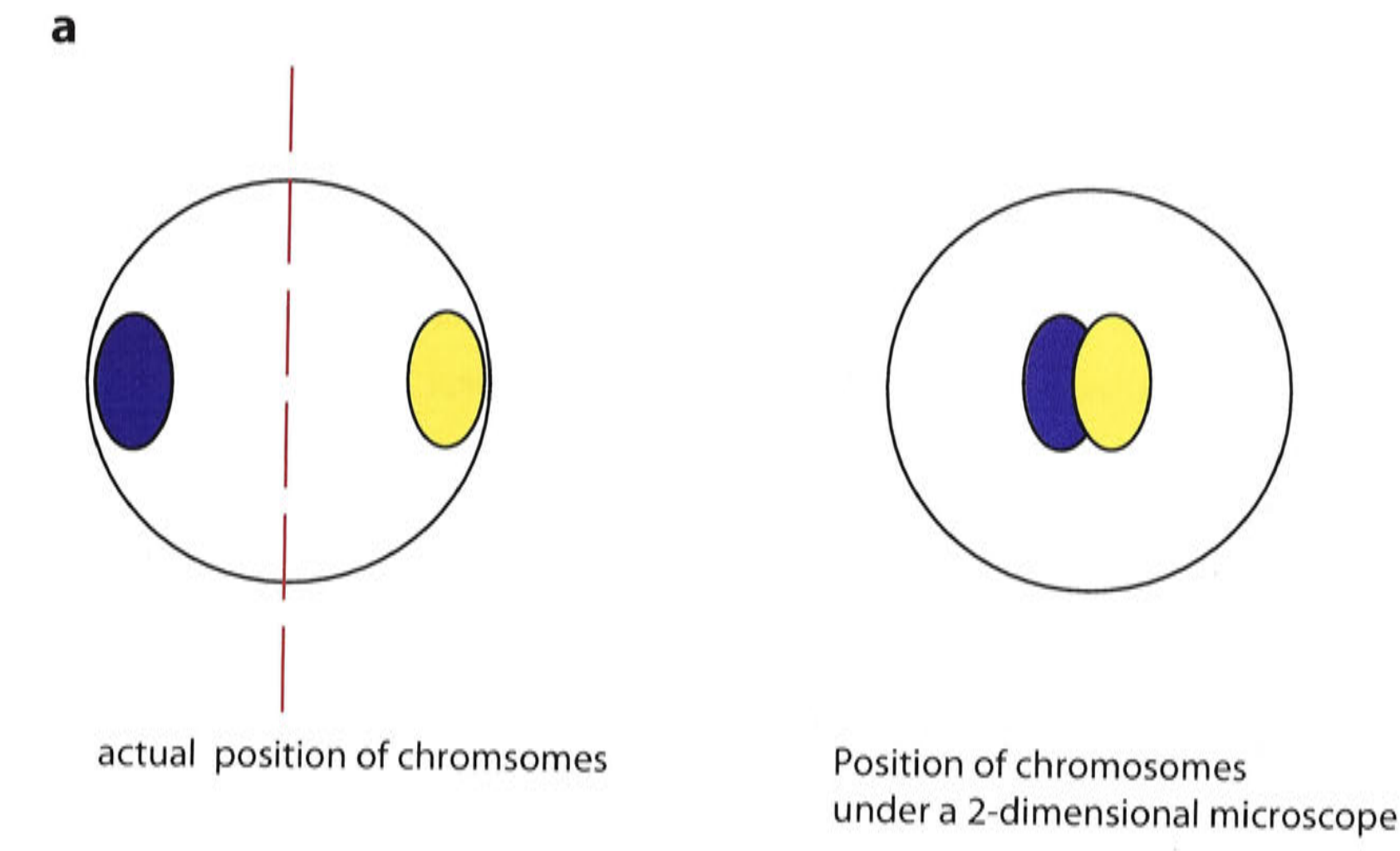


Figure 5.14: Limitations of 2-dimensional microscopy. (a) Depending on angle of the cell, two chromosomes may seem in close association even though they aren't. (b) Two chromosomes next to each at the side of the nucleus is an accurate interpretation of chromosome position. (c) Methanol : acetic acid fixation may collapse the cell altering chromosome associations.

Chromosomes have a non-random position in mouse pachytene cells that is essential for proper segregation of chromosomes and development of spermatozoa (Garanga et al., 2001). Garanga came to this conclusion using methods similar to those of this study, where testes were minced to separate germ cells, which were then put in a hypotonic and fixed in methanol: acetic acid.

Once again the apparent inconsistencies between studies on chromosome arrangement becomes apparent. However, fixing techniques that preserve the three dimensional structure seems essential for the study of chromosome organization in the earlier stages of meiosis.

5.3.2 Characteristics of the marsupial SV

The sex vesicle was studied at meiosis in an American and an Australian marsupial, using chromosome painting to distinguish the X and Y chromosome and an SCP3 antibody to delineate the synaptonemal complex. It was determined that the marsupial SV shared many characteristics with the eutherian SV, such as a balloon shape, position at the periphery of pachytene nuclei and bending of the X chromosome around the Y chromosome. However, in contrast to the eutherian SV, no synaptonemal complex formed between the X and Y, and no pairing between the tammar X and Y chromosome could be demonstrated by painting the tammar X and Y and the shared heterochromatic region. This suggests that the characteristics of the SV such as condensation and peripheral position of the SV do not depend on pairing or SC formation. Important influences in producing SV position and shape in eutherians as well as marsupials could be attachment to the nuclear membrane and formation of the dense plate.

The orientation of the X and Y chromosomes within the SV of the tammar wallaby could be analyzed by observing the position of the heterochromatic region shared between the Xp and Yq using chromosome painting to detect positions of the X, Y and the shared region. The relationship of the two arms of the X and Y chromosomes seemed to be inconsistent. In some SVs a single yellow signal was observed, representing the two heterochromatic regions adjacent to each other (figure 5.15a) and in other SVs there were two yellow regions representing two separate heterochromatic regions (figure 5.15b). I concluded from these results that orientation of chromosome

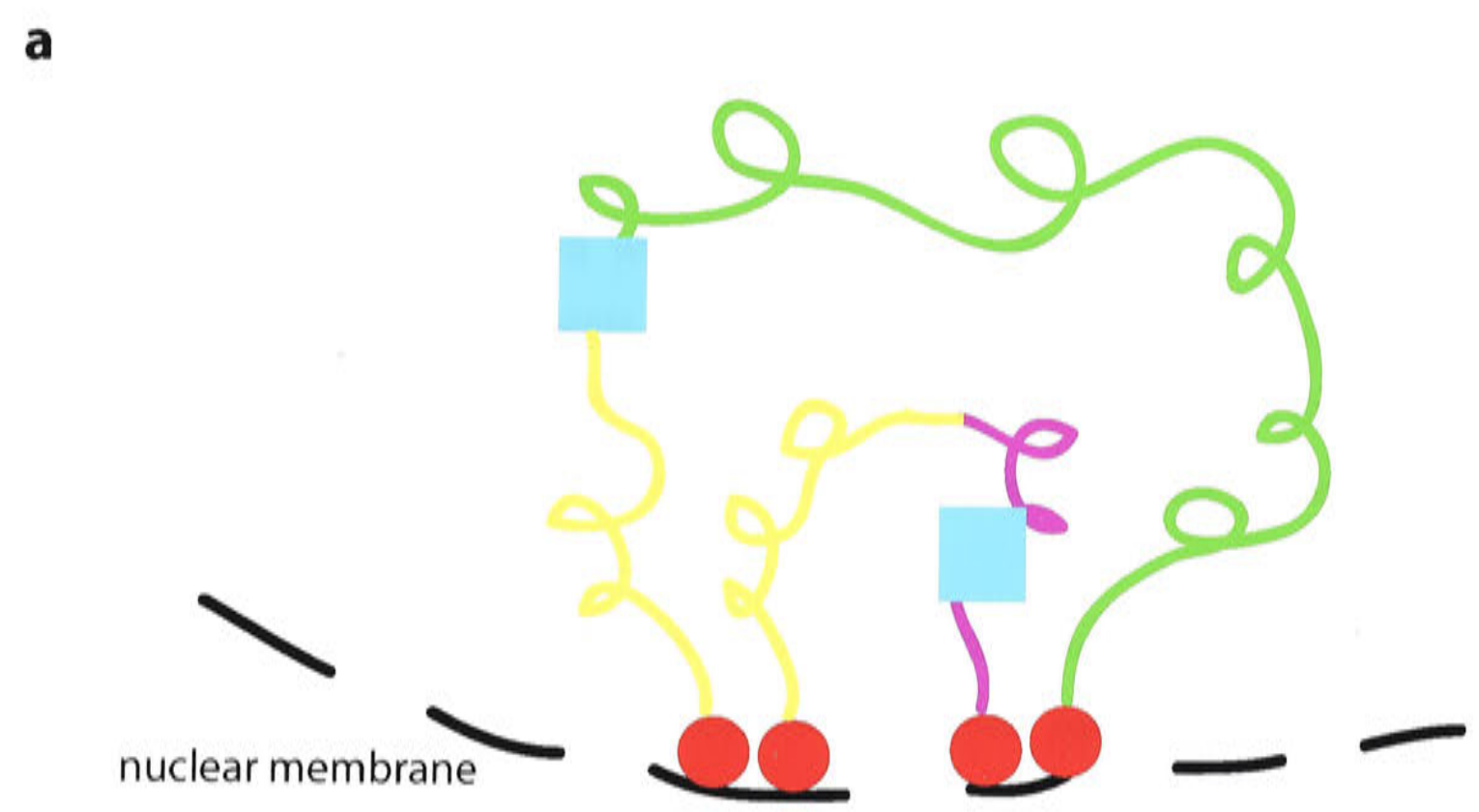


Figure 5.15: X and Y chromosome organization in the tammar wallaby sex vesicle. The X chromosome (green) assumes a horse-shoe shape surrounding the Y chromosome. (pink). In some nuclei (a) the heterochromatic regions of the X and Y (yellow) formed one signal, and in others (b) the heterochromatic regions produced two signals. The attachment of the chromosomes to the nuclear membrane and dense plate therefore does not depend on the relative orientation of the two chromosomes. ■ =centromere, ● =telomeres.

arms between the X and Y chromosomes does not matter, and therefore attachment to the nuclear membrane and dense plate is independent of chromosome arm orientation, and thus of any kind of specific XY pairing.

My results correlate well with the findings of Page (2001), who provided a model by which the axial elements elongate inhibiting the formation of a SC. They found that the dense plate appeared during the progressive thinness of the sex AE and that orientation of the chromosomes in relation to each other did not matter (Page et al., 2002).

Thus the organization and most of the physical characteristics, of the SV are conserved between eutherians and marsupials. However, each has evolved a different method in maintaining the sex vesicle. In marsupials SV formation follows dense plate formation and binding to the nuclear membrane, whereas in eutherians SV formation depends on the formation of the synaptonemal complex.

CHAPTER 6: HISTONES IN MOUSE AND MARSUPIAL SPERMATOGENESIS.

6.1 Introduction

Histones are post-translationally modified to affect chromatin organization, transcriptional activity and play an important role in cell regulation. These modifications include acetylation, phosphorylation, ubiquitination, methylation and ADP-ribosylation (discussed in section 1.6). Variants of the core histones are also found within the cell, each having a specific function in chromatin structure and transcriptional regulation (discussed in section 1.6). Recent studies of a newly – discovered histone variant, H2AZ, have found it to have an important role in *Drosophila* and mouse development (Clarkson et al., 1999, Faast et al., 2001). It has been suggested that the pattern of modifications constitutes a "histone code", which works in unison to promote transcription, condensation or inactivation (Jenuwein and Allis, 2001).

Spermatogenesis is an ideal model system in which to study histone modifications and variants. Spermatogonia A and B undergo mitosis, pass into preleptotene, then undergo meiosis, when the DNA replicates once, and the cell undergoes two separate divisions. This allows observations to be made of chromatin condensation, decondensation, differential packaging and activity. There are stages with high transcription levels, and cell types that are virtually inactive (reviewed by Kleene, 2001). In elongating spermatids, histones are first replaced with transitional proteins and then with protamines. It is now apparent that just prior to histone replacement a series of modifications occurs to different core histones, producing nucleosome instability that allows the DNA to be packaged with protamines. Spermatogenesis is therefore an ideal model to observe the differing roles of histone modifications and variants.

In previous chapters, I demonstrated that a non-random chromosome arrangement is conserved in mammalian sperm, which may set up chromosome domains and expression patterns within the zygote. Imprints may also be carried through the sperm to the zygote to establish parent specific expression patterns. One example is paternal X-inactivation where the paternal X chromosome is imprinted for

inactivation in the embryo. I have focused on observing histone modifications and variants during spermatogenesis in marsupials and mice in the hope of better understanding how chromosome arrangement in sperm may be related to gene function, especially in marsupial paternal X-inactivation. In this chapter, I shall therefore discuss the known role of particular modifications and variants in spermatogenesis and the implications this may have for the zygote.

6.1.1 Histone modifications in meiosis

6.1.1.1 Histone acetylation

In mice, the X chromosome is inactive during prophase 1 of male meiosis, when the X and Y chromosomes form a sex vesicle at the periphery of the nucleus (ch. 5). However, it is not clear whether the mechanism of inactivation at meiosis is the same as for somatic cells, which involves histone deaceylation, and histone variant macroH2A.

There is some evidence for histone deacetylation on the X chromosomes of the desert locust during meiosis. Hyperacetylated histones were detected on autosomes in spermatogonia and spermatocytes (Wolf and Turner, 1996), but facultative heterochromatin showed low histone acetylation staining. Where individual chromosomes could be distinguished the whole X chromosome was underacetylated except for a terminal band (Wolf and Turner, 1996).

However, in *Drosophila* and mice, there are differences between the mechanisms of X-inactivation in somatic and meiotic cells. In *Drosophila* somatic tissue, dosage compensation occurs by hyperacetylation of the single male X chromosome, whereas at meiosis, the X chromosome is not hyperacetylated (Rastelli and Kuroda, 1998).

In eutherian somatic tissue, histones on the inactive X are underacetylated (Jeppesen and Turner, 1993). At male meiosis in mouse, the X chromosome is transcriptionally inactive during prophase 1 (Monesi, 1965) as it is in somatic tissue?. However, using antibodies to acetylated H4, it was observed that the X chromosome is not underacetylated at this stage (Armstrong et al., 1997). This suggests that in mice, too, a different mechanism maintains the inactive state of the X chromosome in somatic and meiotic cells. This result is no longer very surprising, since it is now known that there are specific proteins associated with the sex vesicle (ch. 5), some of which are found only at meiosis. Different strategies may therefore have evolved for somatic and germ cell X-inactivation.

The pattern of histone acetylation changes between different spermatogenic stages. Armstrong and colleagues (1997) used antibody to acetylated lysine 12/8 H4 to demonstrate, in pachytene cells, that histones are uniformally acetylated at these positions (Armstrong et al., 1997). By quantifying the amount of incorporated [H^3]acetate in rat testis, it was demonstrated that [H^3]acetate was incorporated into pachytene cells, indicating acetylation of histones (Grimes and Henderson, 1983).

Acetylation patterns of histones H2A, H2B, H3 and H4 during spermatogenesis were extensively studied by Hazzouri, (2000b) by immunodetection of acetylated histones in paraffin sectioned mouse testis. Figure 6.1a shows that histones were acetylated early in spermatogonia and later in round and elongating spermatids, but not during meiosis in primary spermatocytes. The absence of acetylation in primary spermatocytes was checked by preparing a western blot of chromatin from pachytene cells, which were reported to show no binding of antibodies to acetylated histones, reinforcing the conclusion that histones are not acetylated in pachytene cells. However, these data (which were not presented in the paper), pose some inconsistencies with previously published material, perhaps reflecting technical problems with the penetration of antibodies into the 5μm sections used by Hazzouri (2000b).

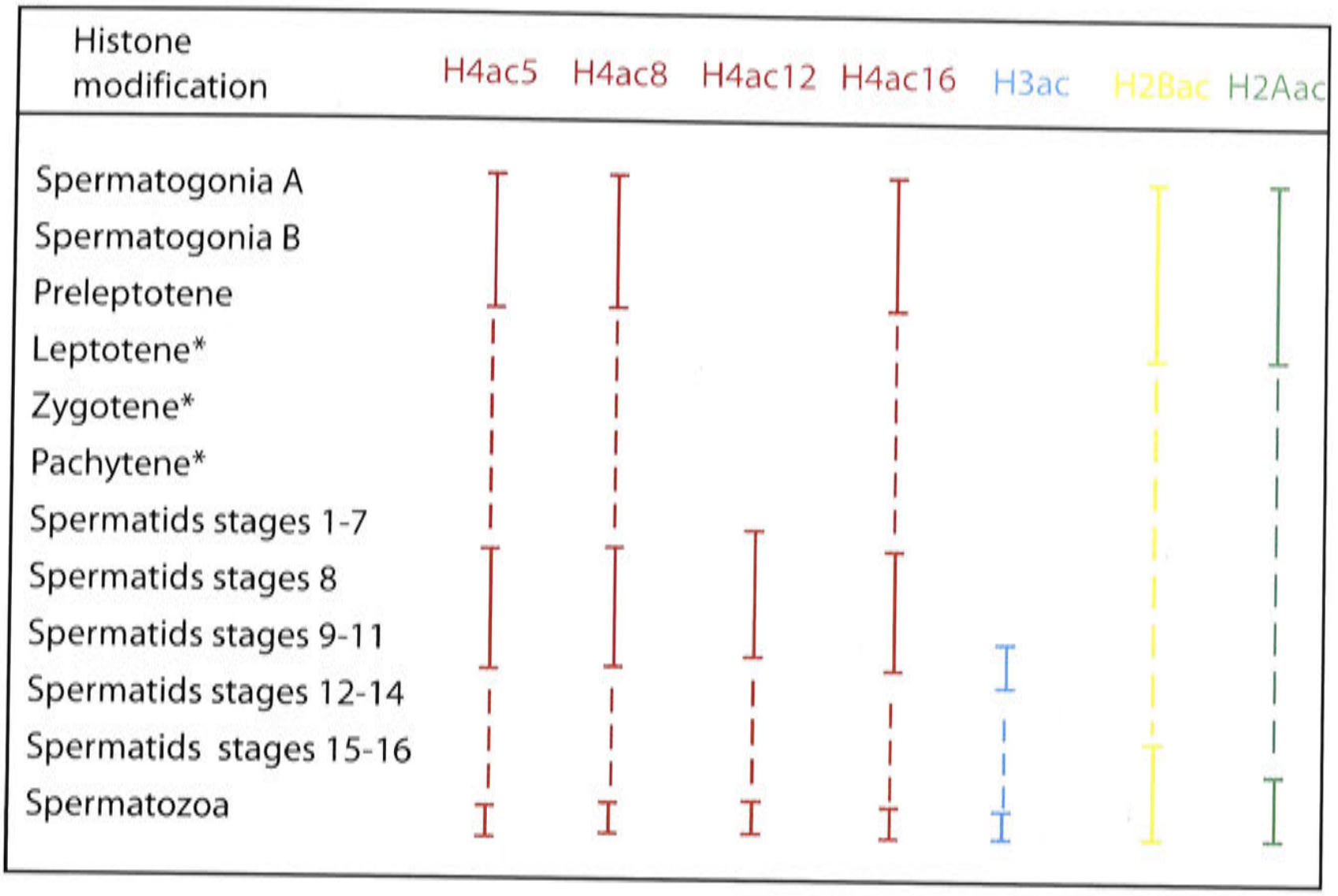
Histones in mouse elongating spermatids are hyperacetylated as part of the histone replacement process (Christensen et al., 1984), weakening nucleosome-nucleosome interactions in preparation for removal and replacement of histones by protamines (Oliva and Dixon, 1991). Acetylated histones then disappear as the protamine packaged DNA condenses through the last steps of spermatid maturation.

These observations show that the acetylation levels are dynamic during meiosis, as might be expected for cells undergoing great changes in gene activity. During the early stages of spermatogenesis, mitotically active cells that are differentiating into meiotic cells have high levels of transcription, and therefore high levels of acetylated histones (reviewed by Kleene, 2001). The acetylated histones observed in the later stages of meiosis represent the repackaging of histones with protamines rather than being an indication of cell transcriptional activity.

6.1.1.2 Histone ubiquitination

Ubiquitination is another histone modification that has been studied in meiosis. Ubiquitinated histone H2A (uH2A) has been detected in spermatogonia using histone immunoblotting, in high levels in pachytene spermatocytes, where it localizes to the sex vesicle, and in elongating spermatids (Baarends et al., 1999) (figure 6.1b). The function

a



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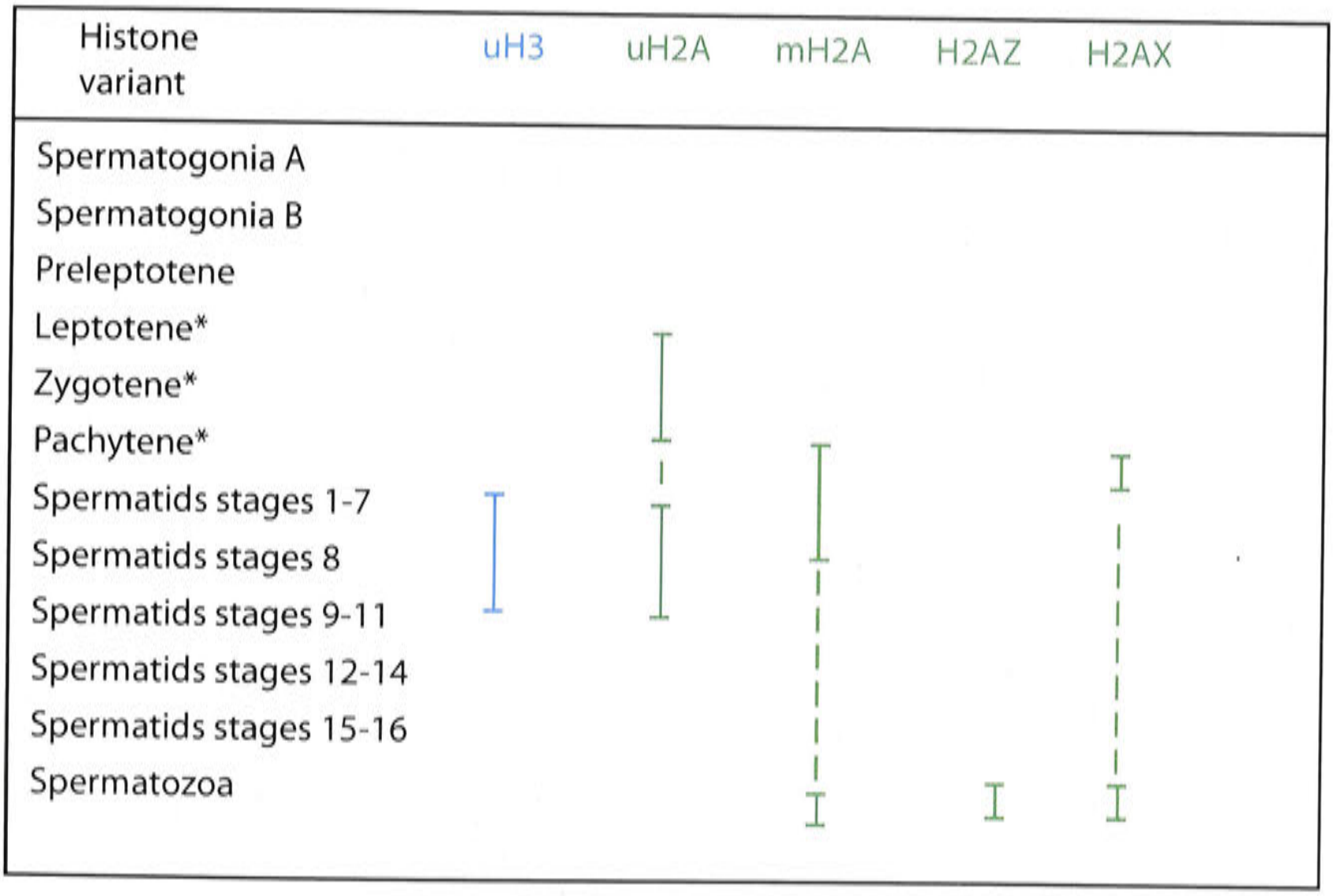


Figure 6.1: Histone modifications and variants in mouse male meiosis. Presence is indicated by solid line, absence by dashed line. (a) Modifications. Histone acetylation is found in spermatogonia and st 1-12 round spermatids. No acetylation was observed in zygotene or pachytene primary spermatocytes. (b) Histone variants. Most histone variants are found in pachytene cells and early stage spermatids. Western blotting has identified some histone variants in sperm. Modified from Hazzouri *et al*, 2000b.

of H2A ubiquitination is not yet known. An effect on nucleosome stability and gene transcription (Li et al., 1993) seems unlikely because the most intense uH2A signal is detected on the transcriptionally inactive SV in pachytene cells (Baarends et al., 1999). However, H2A ubiquitination in elongating spermatids suggests it may play a role in changing nucleosome stability and allowing histone replacement. Thus, ubiquitination may have more than one function:

- As embodied in the idea of a histone code, uH2A may act differently depending on other surrounding modifications.
- Nucleosome stability in the SV may be affected to allow the specific proteins (M31, XY40, XY77) to bind to produce a stable inactivation state.
- Ubiquitination occurring at different amino acid sites may have different functions (cf. methylation of different lysines on H3 have different functions. Methylation at lysine 9 occurs on the inactive X chromosome and therefore seems to be associated with inactive chromatin, whereas methylation of lysine 4 is associated with transcriptionally active DNA).

Histones H3 and H2B also undergo ubiquitination in meiosis and in elongating spermatids (Chen et al., 1998). As for uH2A, the effect of ubiquitination on nucleosome stability would allow histone replacement. Alternatively, the addition of ubiquitin to H2A, H2B and H3 in elongating spermatids may tag them for degradation once the histones have been replaced by protamines (reviewed by Jason et al., 2002).

Thus the modifications normally utilized by somatic cells to allow transcription, are used in meiosis for a different purpose, histone replacement.

6.1.1.3 Other modifications

Other histone modifications have been less extensively studied at meiosis than have histone acetylation. Phosphorylation of histone H3 also occurs in meiosis, and has a role similar to that that in mitosis, where at metaphase I and II histone 3 is phosphorylated, helping to condense the chromosomes and disappears during anaphase (Manzanero et al., 2000).

Methylation of histone H3 has not yet been studied at meisois but as HP-1, a protein that localizes to chromatin through its chromodomain that recognizes methylation of lysine 9 of H3, localizes to the sex vesicle, we can predict H3 methylation at lysine 9 is also present.

6.1.2 Histone variants in spermatogenesis

Different histone variants are also found at different stages of male spermatogenesis. Two histone variants mH2A and H2AZ are of particular interest because of their different and opposite associations with active chromatin in somatic cells (Rangasamy et al., 2003). As discussed in 1.6, macroH2A is a large histone H2A, two thirds of which is a non-histone region. It is associated with heterochromatin (eg. the inactive X chromosome). On the other hand, H2AZ seems to provide a chromatin state open for protein binding.

In male meiosis in mouse, macroH2A localizes to the sex vesicle (Hoyer-Fender et al., 2000, Richler et al., 2000). MacroH2A may be directed to localize to the SV by *Xist* RNA that coats the X chromosome at meiosis (Ayoub et al., 1997). MacroH2A is particularly prevalent in round spermatids, where it localizes to centromeric heterochromatin, and it is eventually detected as a single signal at the chromocentre of round spermatids (Hoyer-Fender et al., 2000). MacroH2A has also been described in elongating spermatids, at the same stages that histone is acetylated and ubiquitinated.

Another recently discovered variant, H2AZ, has also been studied in mouse spermatogenesis. Two-dimensional polyacrylamide gels were used to detect H2AZ in elongating spermatids, and its disappearance during the replacement of histones with protamines (Nickel et al., 1987). Little more is known about the role of H2AZ in meiosis.

6.1.3 Histones in sperm

Although most of the histones in human sperm are lost during spermatid elongation, 15% of the DNA of the mature sperm is packaged in histones. This fraction contains the core histones, including the variants mH2A, H2AZ and H2AX (Gardiner-Garden et al., 1998, Gatewood et al., 1990, Hoyer-Fender et al., 2000). High performance liquid chromatography showed a high level of H2AX (section 1.6), as well as small amounts of H2B, H2AZ, H3 and H4, in human spermatozoa. H3 and H4 were acetylated at the same sites as in somatic cell active chromatin (Gatewood et al., 1990).

Histones in sperm are not randomly distributed, but are associated with specific DNA sequences. Protamine - and histone - associated DNA fractions were separated

and two unique clones were isolated from each type of isolated DNA (Gatewood et al., 1990). Hybridization of these clones back to a western blot containing lanes of nucleohistone, and nucleoprotamine demonstrated, hybridization of the histone DNA clones to only the nucleohistone lane and the protamine DNA clones to only the nucleoprotamine lane. This demonstrated that the same specific sequences are packaged with histones in all sperm.

Separated histone and protamine packaged chromatin fractions have also been probed for β-globin genes (Gatewood et al., 1987). The β-globin gene cluster contains the ε- (embryonic), $^{G}\gamma$-, $^{A}\gamma$- (foetal), δ-, and β-globin (adult) genes, as well as a locus control region. The ε- and γ-globin genes are transcribed in the primitive erythrocytes, but the δ- and β- genes are not expressed in the early embryo. It was found that the 5' end of the γ-globin gene was associated with histones in sperm (Gardiner-Garden et al., 1998), but the δ-globin gene had no histone association in the corresponding region (Gardiner-Garden et al., 1998).

This suggests that the packaging of certain regions of DNA with histones in sperm has a functional significance. Important early transcribed genes could be packaged with histones in the sperm to set up transcription patterns for the zygote and early embryo.

The modification of the histones in sperm may also play an important role in setting up activity domains in the zygote. Histone acetylation patterns are inherited through mitotic cell divisions, so the acetylation patterns of histones in sperm may be retained by the zygote (Jeppesen, 1997). Genes that will be inactive in the embryo may be packaged with hypoacetylated histones, whereas genes important for early transcription are packaged with acetylated histones.

6.1.4 Aims of this study

It is important to discover the degree to which histone variants and modifications are conserved at meiosis, in order to determine their involvement in conserved mammalian functions. No observations have yet been made of histone variants or modifications in marsupials and there are still big gaps and inconsistencies in our knowledge of histone modifications and variants in mouse meiosis. In this study, I have focused on specific histone variants and modifications in order to discover how conserved chromatin structures are in meiosis. I therefore studied acetylated histone,

mH2A and H2AZ in meiosis and spermatogenesis to try and understand the role of histone modifications and variants in chromatin regulation in general, and in X-inactivation in particular.

The pattern of acetylated histone H4, histone macroH2A and histone H2AZ were studied in marsupial and mouse somatic and meiotic cells, in order to establish the patterns of these modifications and variants of histones during spermatogenesis, to determine what patterns are conserved, and to provide clues about their function. For instance, a difference in histone patterns between mouse and marsupials could explain how the imprinted paternal X-inactivation is passed onto the offspring through the sperm.

Histone mH2A was studied in marsupial somatic and meiotic cells and compared to mouse, in order to deduce whether this variant has a conserved role in X-inactivation, despite the phenotypic differences between eutherians and marsupials. As yet, it is not known whether mH2A is associated with the marsupial inactive X chromosome. The newly discovered H2A variant, H2AZ, was also studied in the expectation that the pattern of its presence in meiotic stages may shed some light on how it influences chromatin structure. This essential histone variant has been implicated in actively expressing and differentiating cells.

6.2 Results

Several different antibodies were used to study patterns of modified and variant histones in marsupial somatic and germ cells. These antibodies detected mH2A, H2AZ, and acetylated histone 4. A methylated lysine 9 histone 3 antibody (Upstate technology) was used with limited success.

Two problems were encountered on the first attempts to study histone modification patterns. The first was due to characteristics of the antibody to methylated lysine 9 histone 3, which was designed for use in western blots and assays and not for immunofluorescence. The second problem encountered was the inconsistent results in preparations made using the cytocentrifuge to spread unfixed chromosomes arrested at metaphase. The chromosomes were often damaged beyond recognition and staining by the various antibodies was inconsistent between preparations.

6.2.1 Histone acetylation

6.2.1.1 Histone acetylation in somatic cells

Acetylated H4 of mitotic chromosomes was studied in marsupials, monotremes and a hybrid mouse cell line (*Mus musculus* x *Mus caroli*) that has been used for important work on X chromosome inactivation (Graves, 1982). X-inactivation has previously been studied in this cell line which has an active *M. musculus* X chromosome and inactive *M. caroli* X chromosome(s) (Graves, 1982). This line was used to show that genes on the inactive X are co-ordinately regulated and that repression is controlled by DNA methylation (Graves, 1982). The line also contains many double minutes, which are acentric chromosomal elements seen in variable numbers at mitosis as paired dots. Double minutes result from extra chromosomal gene amplification (Graves, 1984). It was of interest also to discover whether these elements contained acetylated histones.

Mouse B3 metaphase preparations were cytospun onto acetone washed slides, and an antibody to acetylated H4 lysine 8 was used to study acetylation on mouse chromosomes. In 6 metaphase spreads, all chromosomes were labeled apart from centromeres and one chromosome with the size and morphology of the X. (figure 6.2a). In double minutes lysine 8 of H4 was acetylated indicating active transcription (figure 6.2b). Centromeres stained brightly with DAPI but lacked acetylation (figure 6.2c).

The same antibody was used to detect acetylated histone H4 on 4 tammar wallaby metaphase spreads. One whole X chromosome, and the short arm of the other X chromosome were underacetylated, corroborating the results of Wakefield (1997) (figure 6.3). This underacetylation correlates with inactivation of one X chromosome, as well as transcriptional inactivity of the heterochromatic short arm of the other X chromosome.

In 10 female platypus metaphase cells, the poor quality of cytospin preparations made it difficult to identify the X chromosomes. No hypoacetylation was observed that could be ascribed to an inactive X chromosome.

There was striking hyperacetylation of the NOR on chromosome 6 at metaphase (figure 6.4a). The acetylation state of platypus interphase cells was also studied by hybridizing a chromosome 6 paint (figure 6.4b) and an antibody to acetylated H4 (lys 8) to the same interphase cell. It was observed that chromosome 6 was not hyperacetylated at interphase (figure 6.4c).

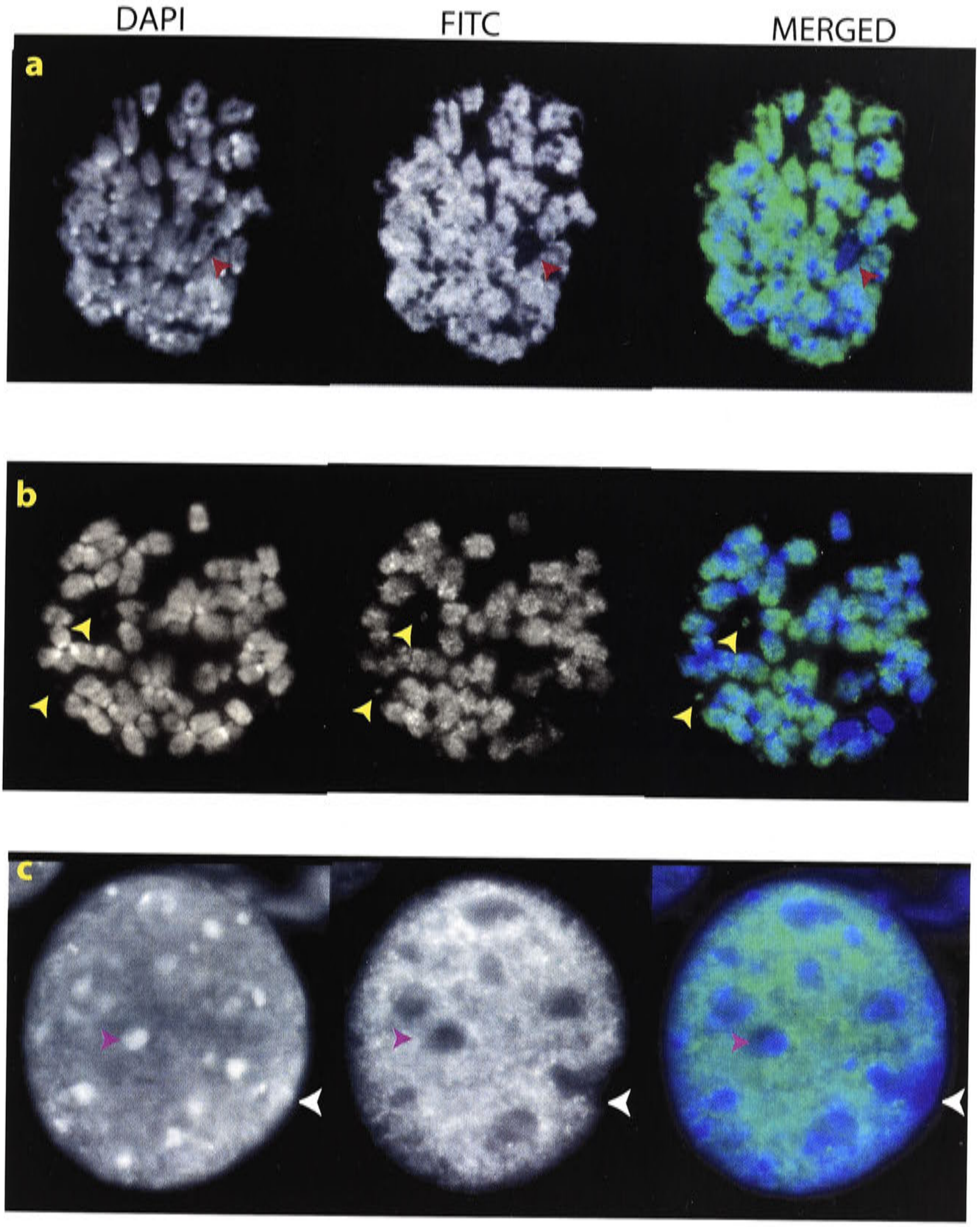
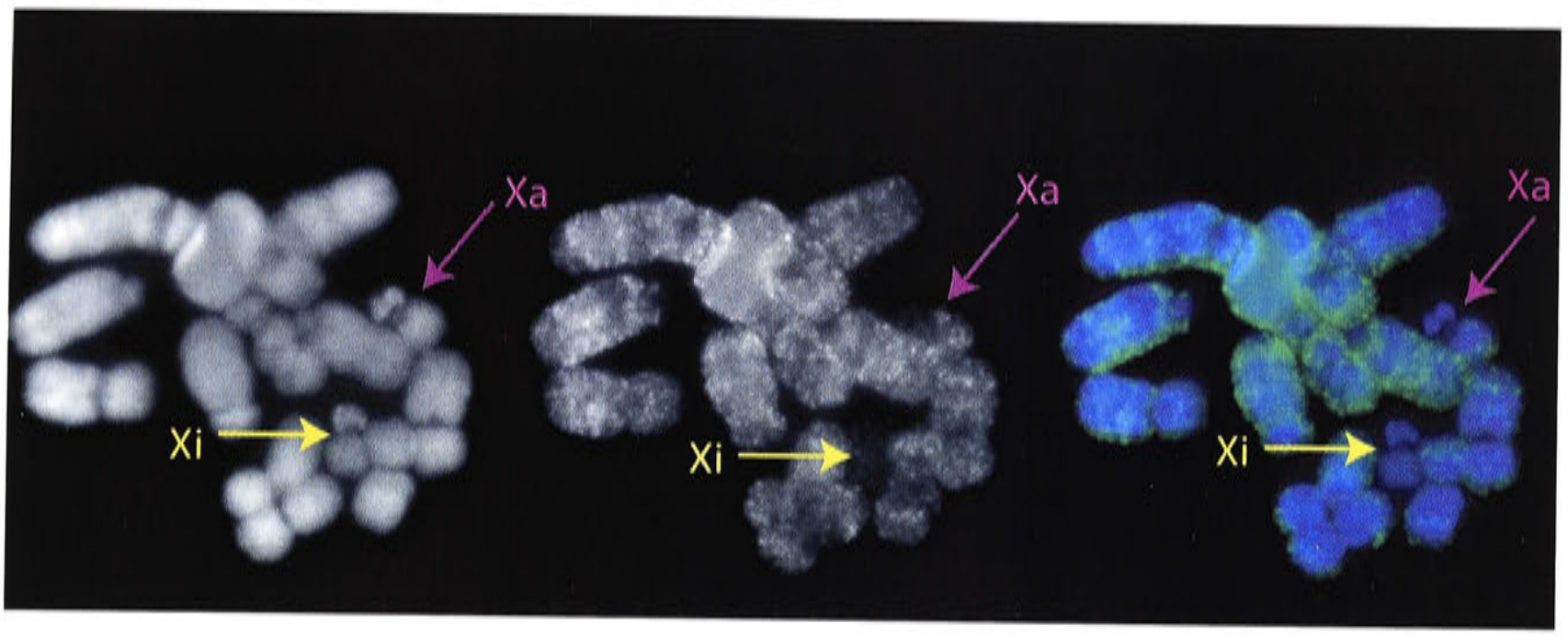


Figure 6.2: Histone 4 (lys 8) acetylation in a mouse B3 cell line. (a) One (possibly 2) X chromosome (➤) is underacetylated. (b) The double minutes (➤) are acetylated, suggesting that they are transcriptionally active. (c) Pericentromeric and centromeric heterochromatin that forms large chromocentres (➤) is underactelyated in an interphase cell. Also a region of the periphery of the nucleus lacks acetylation. This is likely to be the inactive X chromosome (➤).



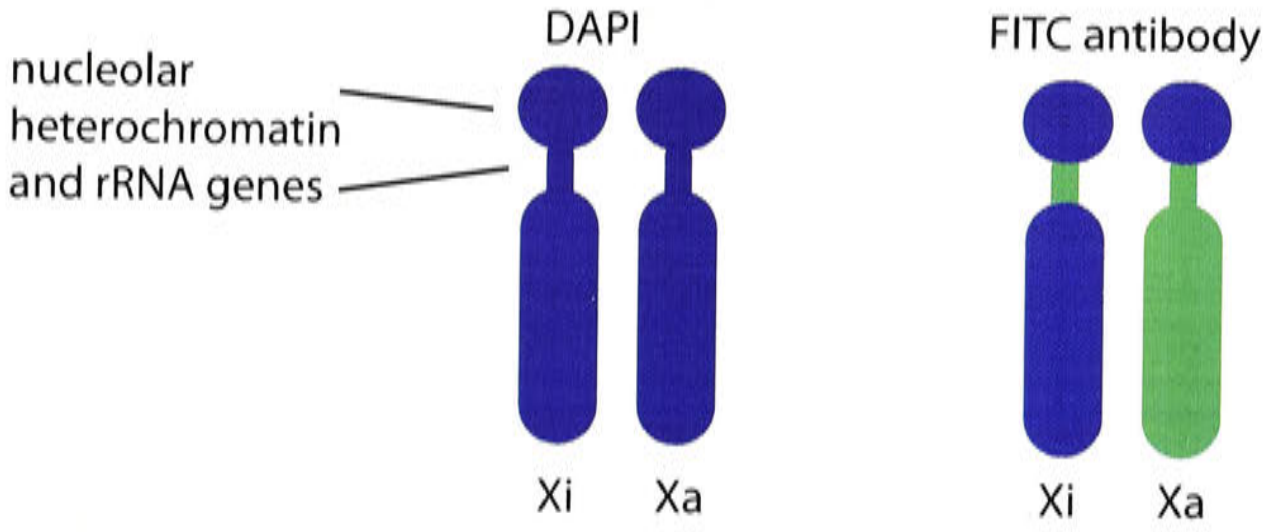


Figure 6.3: Acetylation of the X chromosome in a female tammar wallaby metaphase spread. The tammar wallaby has one underacetylated X chromosome (the inactive X, ➤). The heterochromatic short arm of the active X chromosome is also underacetylated (➤). Green indicates acetylated H4.

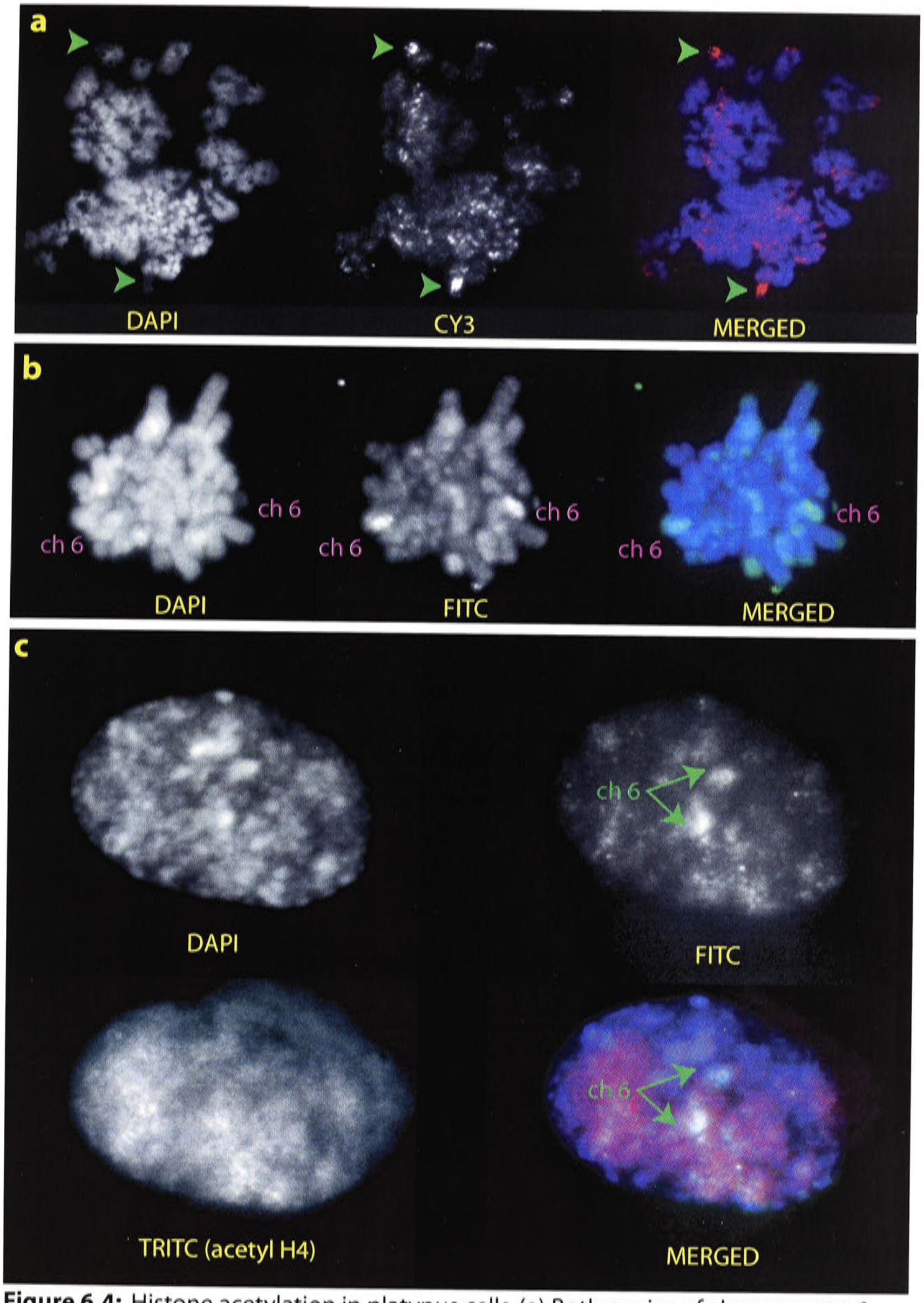


Figure 6.4: Histone acetylation in platypus cells. (a) Both copies of chromosome 6 were hyperacetylated. No differential acetylation of the X chromosome was obvious although the X could not be readily identified in these cytospin preparations (➤). This region represents the actively transcribing NOR of the platypus. (b) Chromosome 6 paint hybridizes with both members of a pair in a metaphase spread. The background reflects low MON1 hybridization. (c) No hyperacetylation was seen on chromosome 6 in platypus interphase cells.

6.2.1.2 Histone acetylation in spermatogenesis

An antibody to acetylated H4 was used to assess chromatin acetylation patterns of spermatogenic cells in mice and marsupials.

Histone H4 acetylation was first examined in histological sections of mouse testis to provide a basis of comparison for acetylation patterns in marsupial testis. H4 acetylation was observed in some spermatogonia, round spermatids and elongating spermatids (figure 6.5). Different acetylation patterns were observed in different regions of seminiferous tubules containing different stages of spermatogenesis. In some seminiferous tubules acetylation was observed only in some spermatogonia and round spermatids (stages 1-4), whereas in other seminferous tubules H4 acetylation was observed only in elongating spermatids (st. 9-11). These results differ from published observations of histone acetylation patterns of surface spread meiotic preparations (Armstrong et al., 1999). However, whether using paraffin sections or surface spread techniques H4 acetylation was never observed at the mouse chromocentre (figure 6.6).

In *S.crassicaudata* (dunnart) testis the acetylation patterns were similar to the pattern observed for mouse, with acetylation in spermatogonia, early round spermatids and in elongating spermatids, but not in spermatocytes. In both mice and marsupials, not all spermatogonia types were acetylated (defined by different patterns of bright DAPI stained heterochromatin) (figure 6.6 and 6.7). Round spermatids had varying degrees of acetylation, which was attributed to the differing efficiency of incorporation of the antibody. This contrasted with published data on mouse spermatogenesis that demonstrated consistent histone acetylation staining in round spermatids (Hazzouri et al., 2000b). However, round spermatids had a consistent H4 acetylation pattern when prepared by surface spreading techniques. In marsupial round spermatids, no chromocentre was observed and H4 acetylation was distributed throughout the cell (figure 6.7). This suggests that the lack of acetylation at the mouse chromocentre reflects the heterochromatin present rather than a specific function. H4 acetylation was also present around stage 9 in elongating spermatids in dunnart and mouse, a stage of mouse spermatogenesis at which histones are known to be acetylated in the preparation for their replacement with protamines.

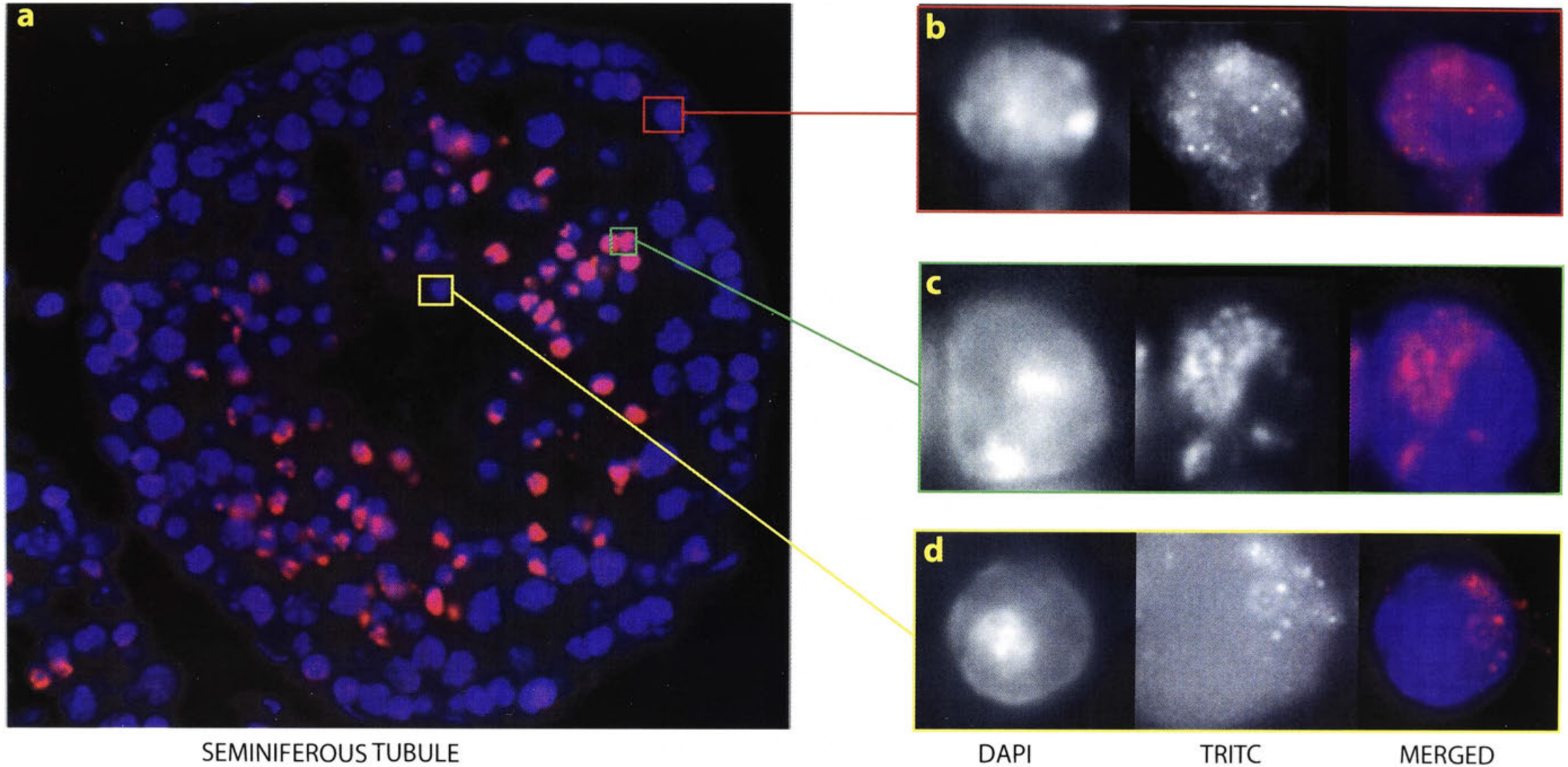


Figure 6.5: Histone 4 acetylation patterns in cells of mouse seminiferous tubule stained with DAPI. a) Transverse section through a seminferous tubule. (b) Labeling spermatogonia with antibody against acetylated H4. One type of spermatogonia contained acetylated histones, but DAPI staining did not distinguish types of spermatogonia. c) Early round spermatids labeled with antibody to acetylated H4. (d). Histone acetylation levels were observed to be inconsistent in stage 8 round spermatids. This is an artifact of using sections, as my surface spread techniques showed a consistent signal for acetylated H4 in all round spermatids.

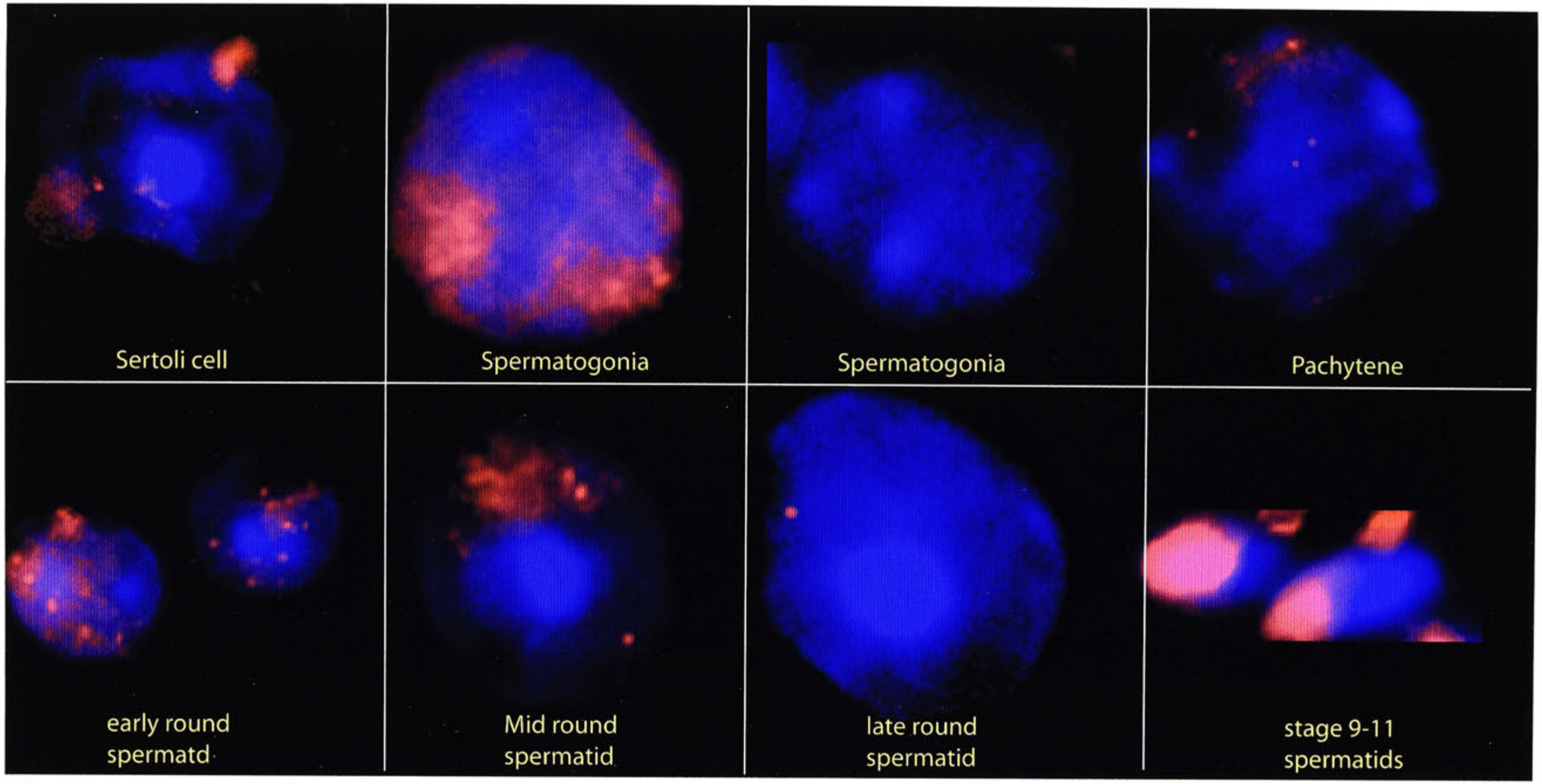


Figure 6.6: Histone acetylation patterns of different cell types from mouse testis sections detected by antibody to acetylated H4. Sertoli cells had no acetylation. Only one spermatogonia contained acetylated H4. Pachytene cells were negative, but early round spermatids and st. 9-11 spermatids all had acetylated chromatin. However, late round spermatids were negative. In all round spermatids the chromocentres were never acetylated. Magnification x 630.

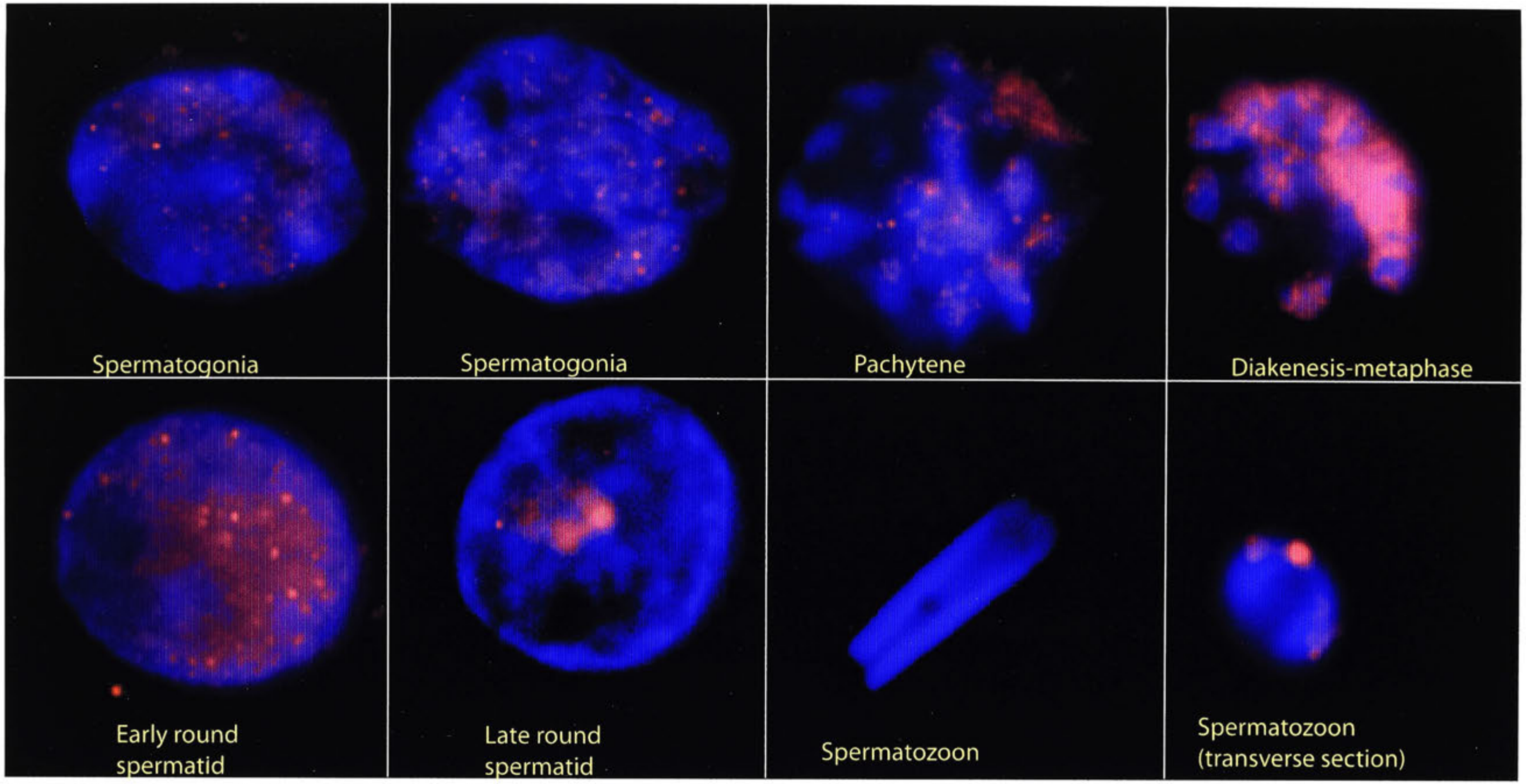


Figure 6.7: Histone acetylation in different cell types from *Sminthopsis crassicaudata* testis sections stained with an antibody to acetylated H4. The pattern was nearly identical to that seen in mouse, with acetylation in some spermatogonia, very little or no acetylation in pachytene cells and differential acetylation in round spermatids. Spermatozoa had no acetylation, except for a thin layer of acetylated histones only observed in tranverse sections, which may correspond to a localized histone region previously reported (Soon *et al*, 1997).

6.2.2 Histone MacroH2A

6.2.2.1 MacroH2A in marsupial and monotreme somatic cells

Sminthopsis douglasi (Julia creek dunnart) and platypus fibroblasts were grown on slides, fixed *in situ* with 4% paraformaldehyde and stained with mH2A antibody. Julia creek dunnart cells were used as they contained normal chromosome numbers. The laboratory cell lines of *Sminthopsis crassicaudata* contain translocations and abnormal chromosome numbers and therefore, were not used.

A light mH2A background staining was observed throughout the nucleus of male and female *S.douglasi* interphase cells, similar to that observed in eutherian somatic cells (Jeppesen and Turner, 1993). In female *S.douglasi* cells a bright signal for mH2A formed a macro chromatin body (MCB) in 70-80% of cells with normally one signal, but occasionally two signals. This MCB was normally found outside the cell nucleus (figure 6.8). Even in cells in which the signal appeared to lie within the nucleus, 2-D analysis made it impossible to determine whether the signal was inside the nucleus or sitting above it in the cytoplasm. Therefore I was unable to determine whether this immunostaining represented the inactive X chromosome or to a cytoplasmic body such as the centrosome. My observation that in male *S.douglasi* cells anti-mH2A produced mH2A signals at the same frequency inside and outside the cell nucleus must mean that the MCB was not the inactive X chromosome (figure 6.8). It seems, therefore that mH2A localizes to the centrosome of marsupials but not to the marsupial inactive X chromosome.

In the platypus cells stained with mH2A antibody, there was slight mH2A staining throughout the cell nucleus, but no MCB was observed either outside or inside the cell nucleus (figure 6.8).

6.2.2.2 Histone MacroH2A in mouse and marsupial spermatogenesis

MacroH2A has previously been observed to play a role in X-inactivation in mouse spermatogenesis, and therefore I wanted to see if this role is conserved in marsupial spermatogenesis. The distribution of mH2A was studied in 56 mouse and marsupial testis cells prepared by surface spreading or sectioning, using an antibody to rat mH2A.

In mouse, results differed slightly depending upon the method used. When a surface spreading technique was used, mH2A was detected at the chromocentre of

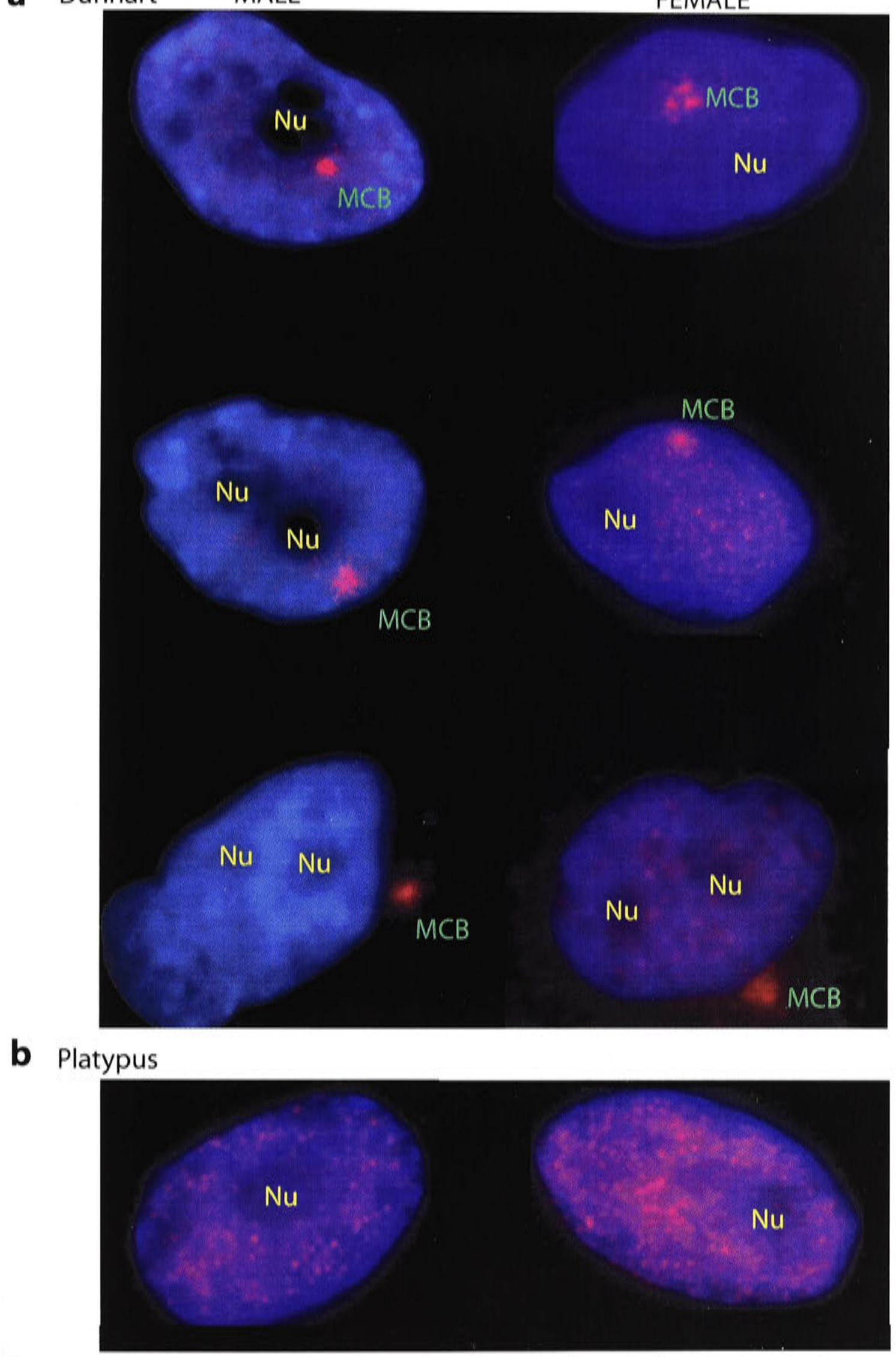


Figure 6.8: MacroH2A in female and male *Sminthopsis douglasi* and platypus interphase fibroblast cells. (a) In female and male cells one MCB signal at the centre or at the periphery of the nucleus was observed. As the MCB is present in males, the signal cannot be the inactive X chromosome. Signals in both males and females were observed outside the nucleus suggesting that it represents the centrosome. (b) No MCB was present in female or male platypus nuclei. All cells had a general staining throughtout the nucleus. The dark region in each cell is the nucleolus.
MCB = Macro Chromatin Body; Nu = Nucleolus.

Sertoli cells, which were recognized by a large faintly stained nucleus with a DAPI stained chromocentre. No mH2A was detected in spermatogonia or early spermatocytes, but in pachytene cells, mH2A localized to the heterochromatic regions, including the sex vesicle, which is recognized through its peripheral position within the nucleus. In round spermatids, the anti mH2A antibody localized to centromeric regions, which coalesced to a single signal at the chromocentre as they matured. No mH2A was observed in any elongating spermatids or spermatozoa (figure 6.9).

A similar pattern was observed in paraffin sections of mouse testis except that the chromocentre was not strongly labeled (figure 6.10). This may be because the centrally located chromocentre is much less accessible in sections than it is in surface spreads. However, it was easy to observe the localization of mH2A to the sex vesicle (SV) in mouse pachytene cells, because it is positioned at the periphery of the nucleus (figure 6.10b-c).

The antibody to histone mH2A was used to study mH2A distribution in cells of tammar wallaby and dunnart testis. Staining with mH2A was much clearer than for mouse because there was little constitutive heterochromatin in marsupial cells. A MCB was observed at the periphery of pachytene cells in the tammar wallaby and the dunnart using surface spreading techniques. In both species the heterochromatic SV could be identified in pachytene cells by its differential DAPI staining, so it could be determined that the MCB forms on the sex vesicle in both marsupial species (figure 6.11). This was confirmed using chromosome paints to identify the sex chromosomes in pachytene cells that had been stained by antibody to mH2A. The tammar Y chromosome paint and the dunnart X chromosome derived probes were used due to the quality of the probe. The tammar wallaby Y chromosome paint was hybridized onto cells that had previously been hybridized with mH2A. In these cells the chromosome paint signal and the mH2A co-localized, confirming localization of mH2A at the SV (figure 6.12a). X chromosome dunnart paint was used to identify the X in dunnart pachytene cells, and again the signal co-localized with the MCB. Thus in both marsupial species, a MCB is found on the SV (figure 6.12b).

MacroH2A could also be found in marsupial round spermatids (figure 6.12 and 6.13), although in the absence of a strongly heterochromatic chromocentre, there was no strong MCB at the centre.

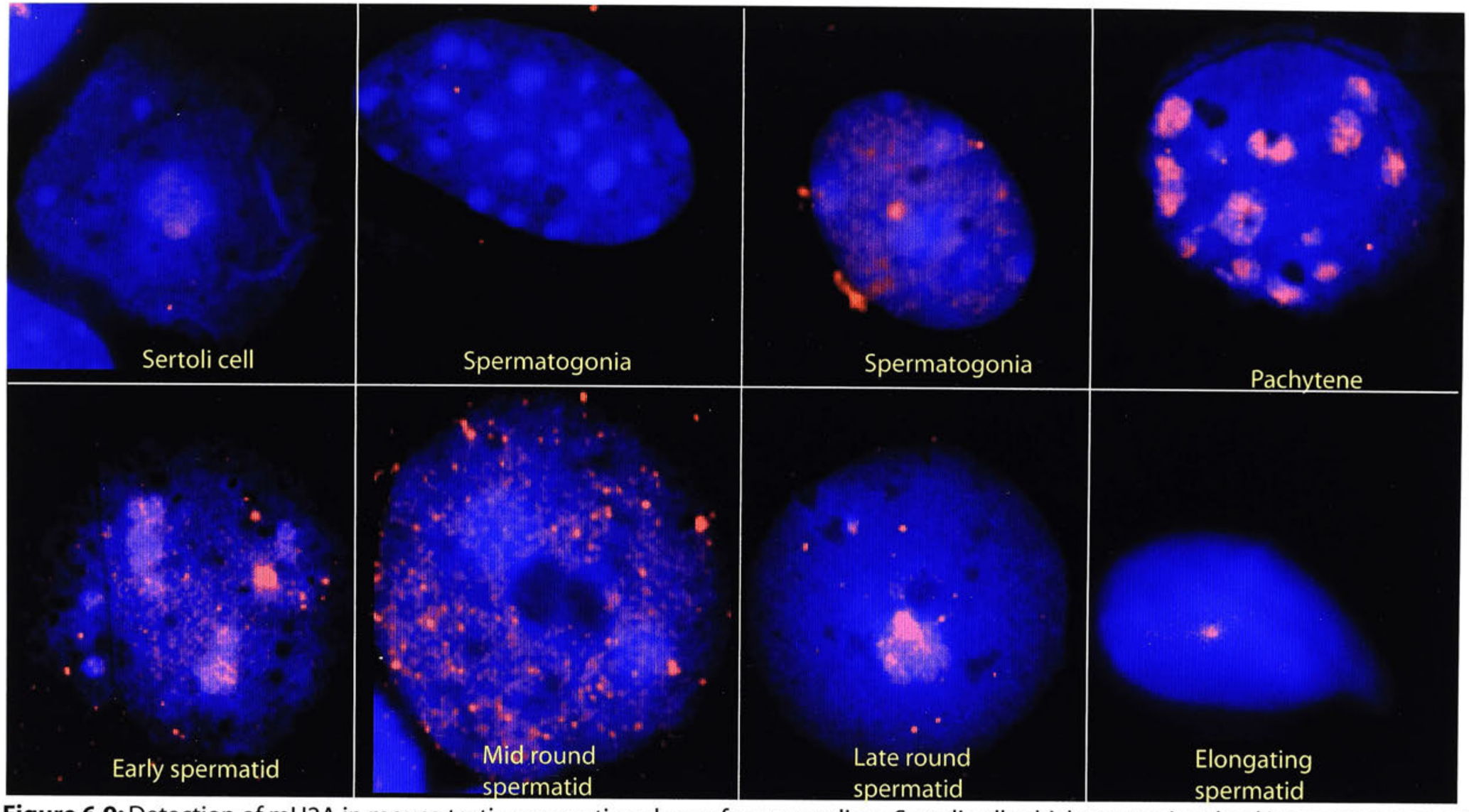


Figure 6.9: Detection of mH2A in mouse testis preparations by surface spreading. Sertoli cell, which are not involved in spermatogenesis, had mH2A localization to the chromocentre.mH2A was located at the SV and heterochromatin in pachytene cells. mH2A localizes to centromeres in late round spermatids eventually producing one signal at the chromocentre. There is no mH2A localization in elongating spermatids (bright spot in elongatinf spermatid repesents dirt).

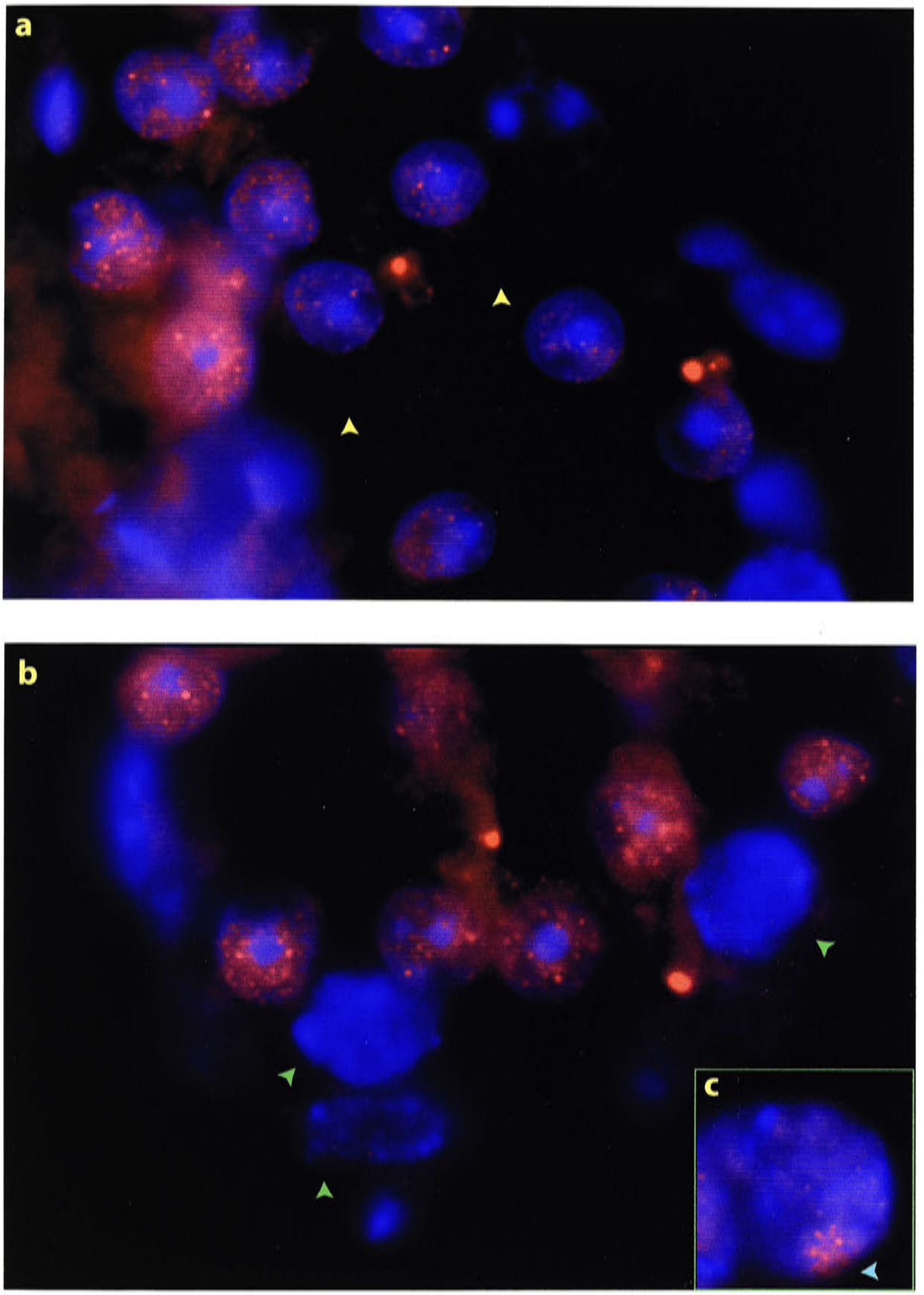


Figure 6.10: MacroH2A in sectioned mouse testis. (a) mH2A was detected in round spermatids, but not in chromocentres, where mH2A localizes in surface spread preparations (➤). (b) Different types of spermatogonia lack mH2A localization, but the types cannot be identified though DAPI staining (➤). (c) The sex vesicle in mouse pachtyene cells is covered in mH2A (➤).

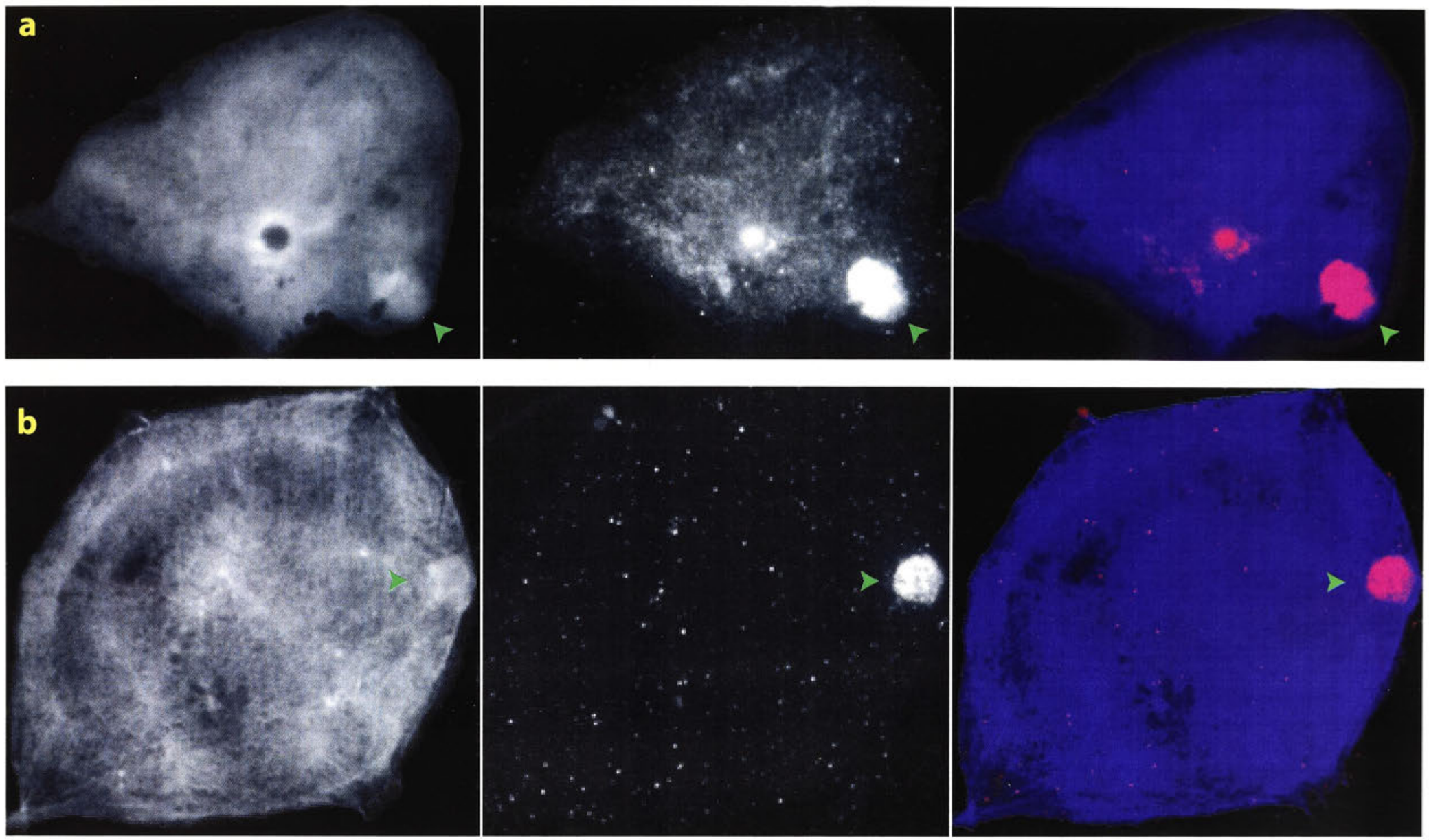


Figure 6.11: MacroH2A in the sex vesicle in tammar wallaby (a) and dunnart (b) pachytene cells. The SV (➤) could be identified by its differential DAPI staining, which was strongly stained by antibody to m H2A.

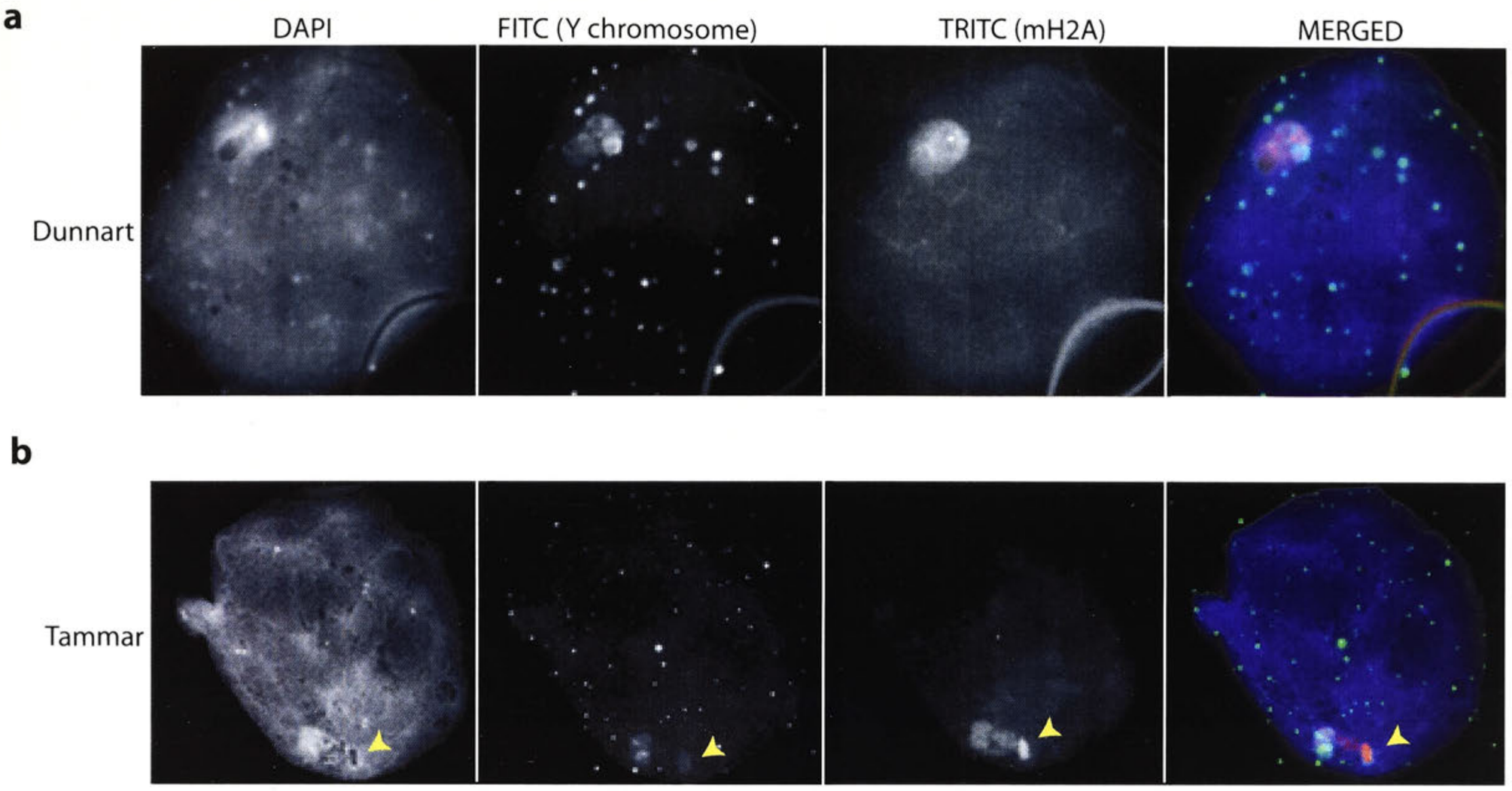


Figure 6.12: The sex chromosomes co-localize with mH2A in marsupial pachytene cells. (a) The Y chromosome (FITC, green) co-localizes with mH2A (TRITC, red) in tammar wallaby pachytene cells. (b) The X chromosome (FITC, green) co-localizes with mH2A (TRITC, red) in dunnart pachytene cells. False signals can be seen where antibody has collected in regions of DNA degradation (➤).

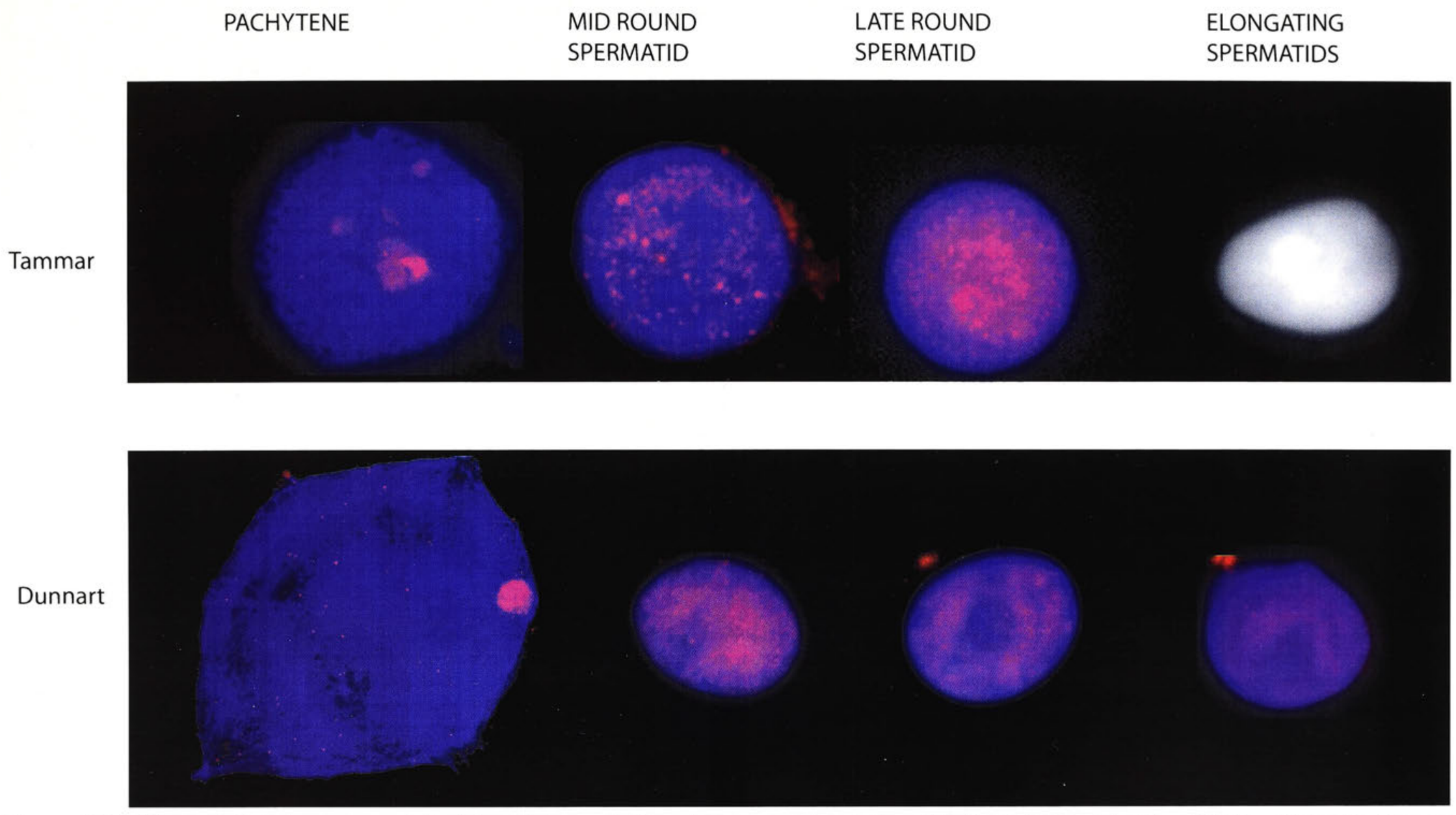


Figure 6.13: MacroH2A in tammar wallaby (a) and dunnart (b) surface spread meiotic cells. Pachytene cell with mH2A localizing to the sex vesicle. Different stages of round spermatids showing the gradual loss of mH2A from the cell.

6.2.3 Histone H2AZ and its relationship to other modifications and variants

The distribution of the newly discovered variant histone H2AZ, which preferentially associates with active chromatin, was observed in 50 cells of mouse and marsupial testis. An antibody against H2AZ was developed in rabbit and supplied by Doctor David Tremethick from the John Curtin School of Medical Research, The Australian National University.

6.2.3.1 mH2A and H2AZ in cells of the mouse testis

To determine the relative locations of mH2A and H2AZ, antibodies were tagged with different flourochromes and applied to the same surface spread mouse cells. The pattern was strikingly complementary. No H2AZ staining was observed in spermatogonia. Pachytene cells showed localization for both mH2A and H2AZ, but mH2A was found to co-localize only with heterochromatin (figure 6.14), whereas H2AZ stained the whole nucleus except for the heterochromatic regions. In pachytene cells the SV was enriched in mH2A but lacked any H2AZ staining (figure 6.14).

Round spermatids stained for both mH2A and H2AZ showed strong mH2A staining over the chromocentre, and H2AZ staining over the rest of the nucleus. Again, H2AZ was not present in regions enriched with mH2A (figure 6.15). Directly adjacent to the mH2A signal a small region of H2AZ enrichment was detected (figure 6.15) which lacked mH2A. H2AZ was present in elongating spermatids up until stage 10-12, when it disappeared (figure 6.16), and no mH2A could be detected in elongating spermatids.

6.2.3.2 H2AZ and acetylated H4 (lysine 8) in mouse testis.

The relationship between H4 acetylation and H2AZ in cells of the mouse testis was studied by staining with an H4ac lys 8 antibody in conjunction with a H2AZ antibody. Acetylated histone 4 was detected in pachytene cells, round and elongating spermatids (section 6.2.2). In pachytene cells both H2AZ and H4ac (lys 8) were found throughout the nucleus, except on heterochromatin. The only difference in H4ac (lys8) and H2AZ distribution was on the SV, which contained acetylated H4 but little detectable H2AZ (figure 6.17a).

In round spermatids at all stages H4ac (lys 8) staining was seen throughout the nucleus except for the chromocentre (figure 6.17). In mid to late round spermatids a region enriched in H2AZ was observed adjacent to the chromocentre that did not stain

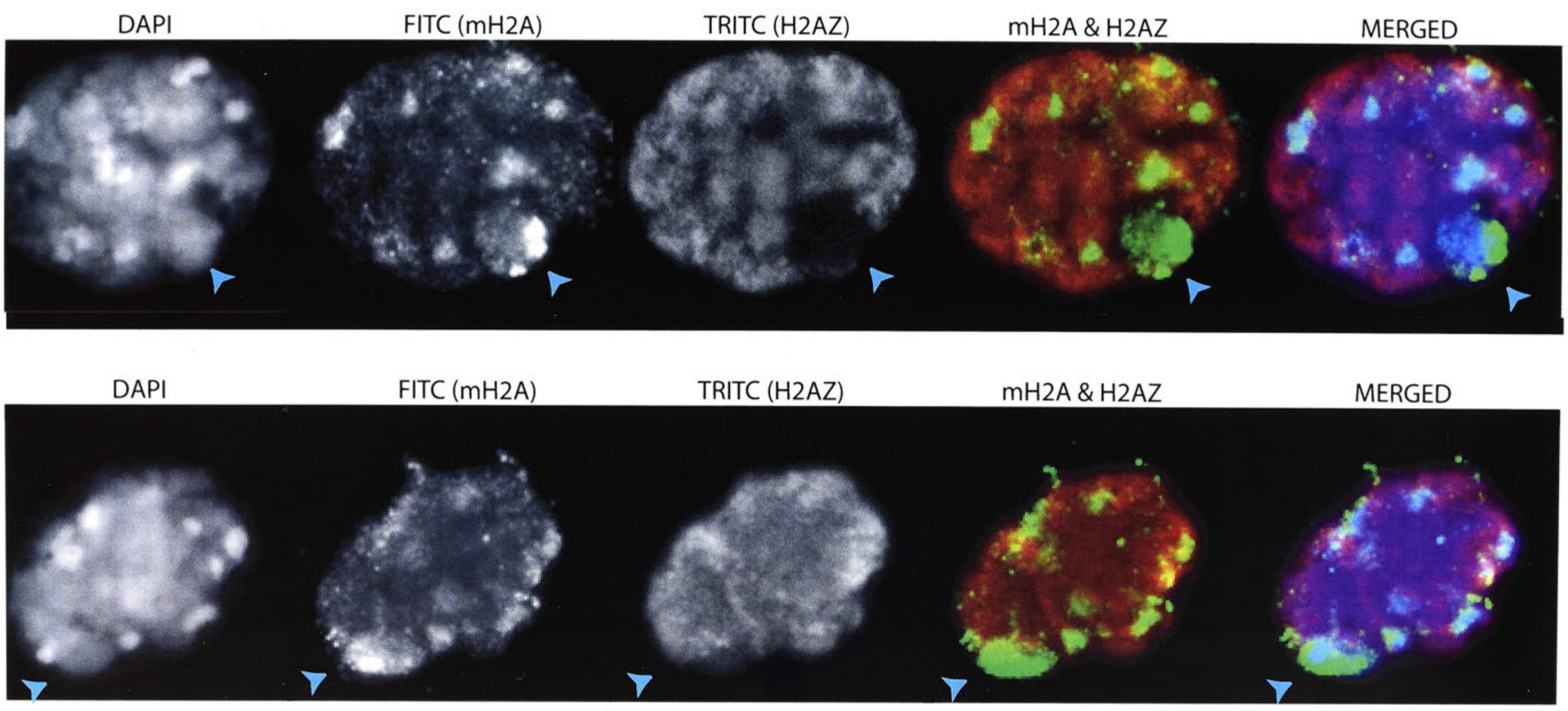


Figure 6.14: Staining of two mouse pachytene cells (upper and lower series) with DAPI (blue) mH2A (FITC, green) and H2AZ (TRITC, red). H2AZ and mH2A do not co-localize. The heterochromatin (including the SV) is packaged with mH2A, whereas the rest of the cell is packaged with H2AZ. The sex vesicle is enriched for mH2A and lacks any H2AZ staining (➤).

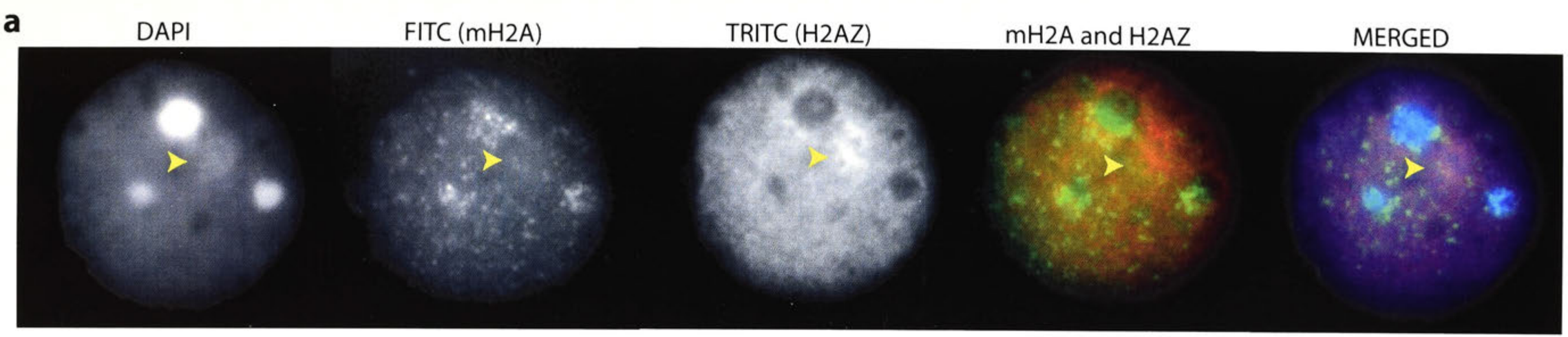


Figure 6.15: Triple staining of two mouse spermatids with DAPI (blue), mH2A (FITC, green) and H2AZ (TRITC, red) (a) mid stage (3-6), and mouse late (b) round spermatid (st. 6-8). Regions enriched in mH2A (centromeres and chromocentre) are not stained with H2AZ. DAPI - dull region is enriched in H2AZ (➤)

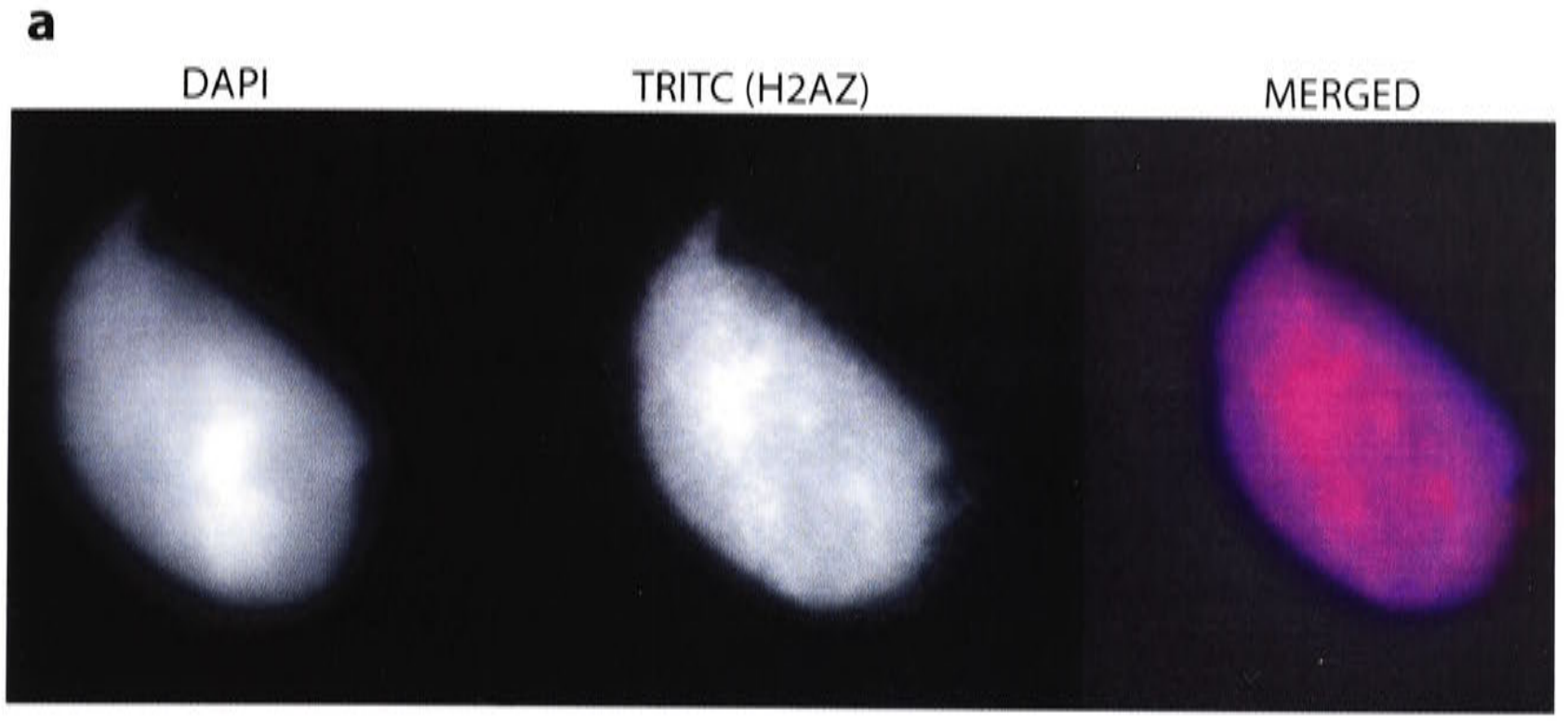


Figure 6.16: H2AZ in mouse elongating spermatids. (a) DAPI staining of a st 9-11 elongating spermatid. (b) No H2AZ or mH2A were found in spermatids from st. 12 onwards.

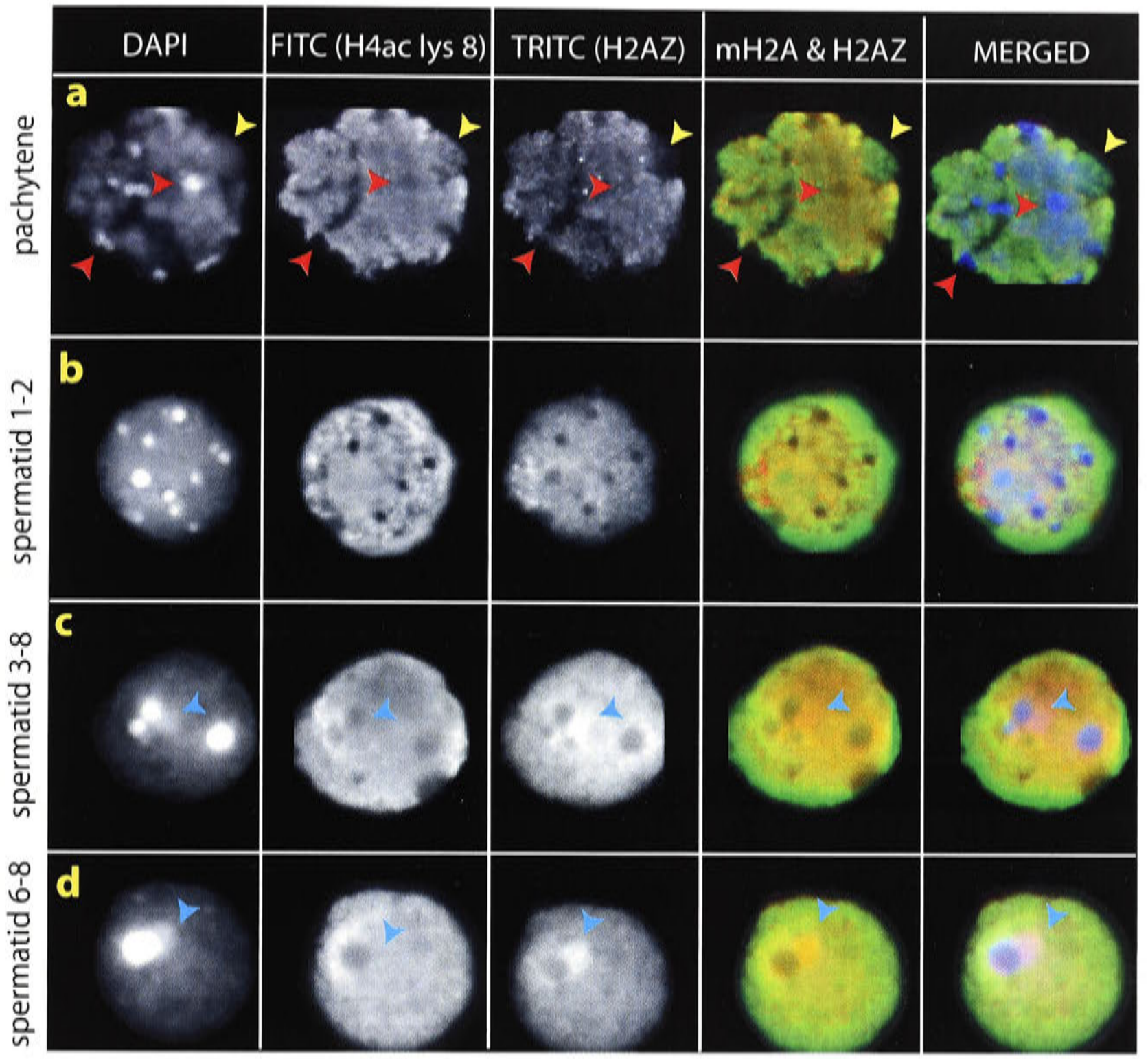


Figure 6.17: Double immunohistochemistry for acetylated H4 and H2AZ in mouse spermatogenesis. (a) In pachytene cells, H2AZ and acetylated H4 was absent from heterochromatin (➤). The sex vesicle is acetylated, but H2AZ is absent (➤). (b-d) Different stages of round spermatids, stages 1-2 (b), stages 3-8 (c) and stages 6-8 (d). H2AZ and acetylated H4 are absent at the chromocentre. A small enriched region (➤) of H2AZ lies adjacent to the chromocentre.

for H4ac lys 8 (figure 6.17c-d) Thus H2AZ and H4ac do not co-localize in this region. Spermatozoa (spermatogenic stages 13-16) were not stained by either H2AZ or H4ac (lys 8), confirming the specificity of the detection.

6.2.3.3 H2AZ in marsupial and mouse testis sections

Antibody to H2AZ was used to observe the pattern of H2AZ distribution in sections of mouse *S.crassicaudata* testis. Difficulties were encounted in getting the antibody to penetrate the paraffin sections. Various procedures incorporating pronase, proteinase K and pepsin digestion were used with inconsistent success, but some results were obtained. H2AZ was detected in mouse pachytene cells, round spermatids (spermatogenic stages 1-8) and in early elongating spermatids (spermatogenic stages 9-12) (figure 6.18). H2AZ was not present in heterochromatic regions or the SV in mouse pachytene cells. In dunnart round spermatids no shaded region or chromocentre was visible after DAPI staining, but there did seem to be a correlation between H2AZ and DAPI bright regions (figure 6.19).

6.2.4 Confocal microscopy of cells stained with H2AZ and H4ac

Two-dimensional microscopy posed two problems in interpreting histone staining in mouse spermatogenic cells. The first was that in pachytene cells, the SV could not always be observed in the nucleus because its position was sometimes beneath the nucleus, so that the absence of H2AZ signal at the SV would be masked by H2AZ staining from the rest of the nucleus. The second problem was that the resolution of two-dimensional microscopy limited the ability to be sure that the H2AZ positive region adjacent to the DAPI bright region, lacked H4 acetylation. To determine whether these interpretations were indeed correct, a confocal microscope was used to view the same preparations. Confocal microscopy offers the advantage that many thin sections of a cell can be observed to create a three dimensional picture of the cell.

Confocal microscopy of 3 mouse pachytene cells showed that the SV stained brightly for H4ac (lys 8), but lacked H2AZ staining in the same focal plane but corroborating the results from 2-dimensional analysis (figure 6.20).

Confocal microscopy on 3 round spermatids revealed a region with no H4ac lys 8 staining, but enriched in H2AZ. The H2AZ enriched region did co-localize with the DAPI stained region (figure 6.21). Furthermore there was a small H2AZ dot in the

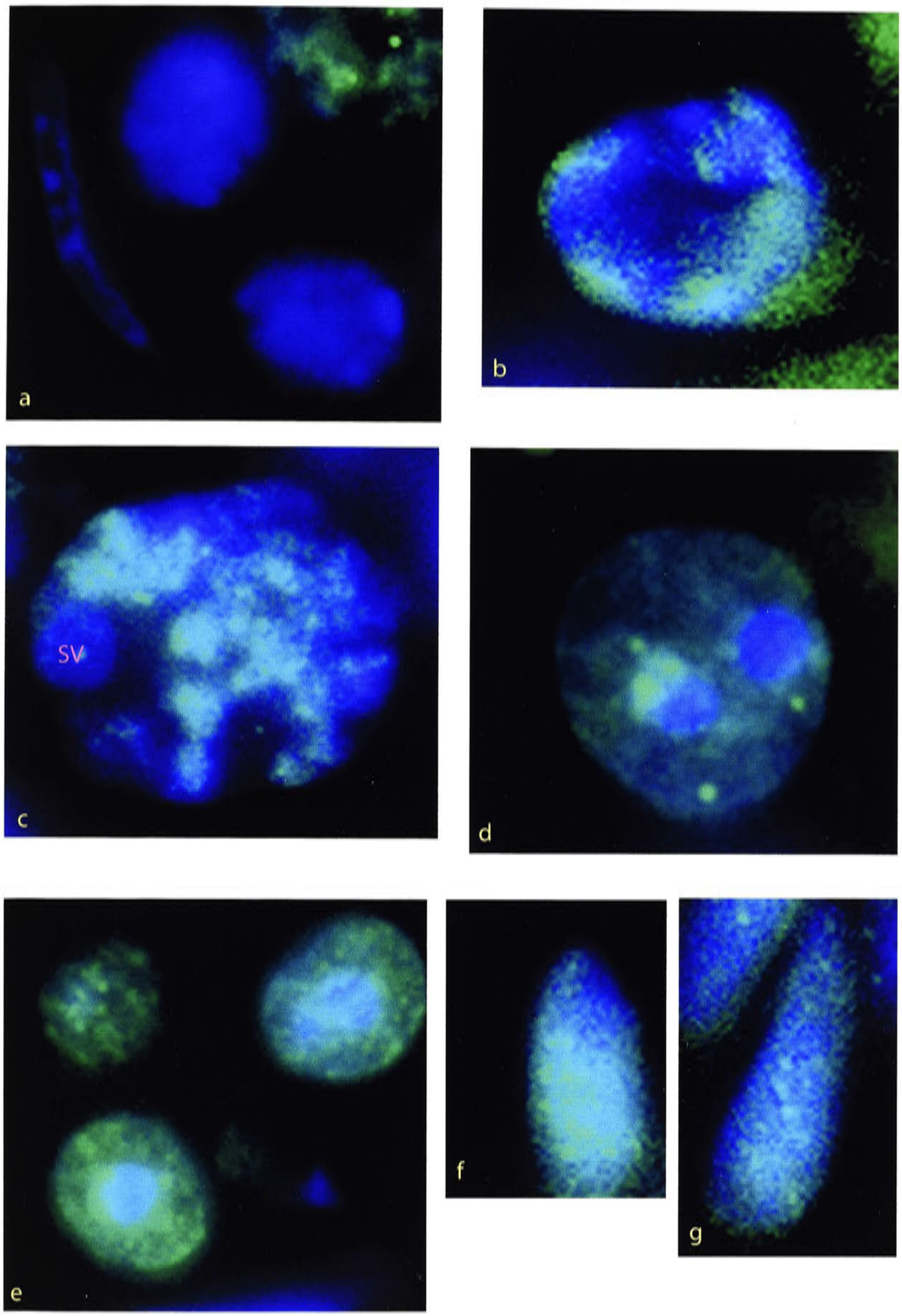


Figure 6.18: H2AZ in cells of sectioned mouse testis. (a) One leydig cell and two spermatogonia. (b) Spermatogonia B or preleptotene cell. (c) Pachytene cell showing an absence of H2AZ staining on the SV. (d) Mid round spermatid. (e) Late round spermatids. (f) Stage 9 spermatid. (g) Stage 11 spermatid

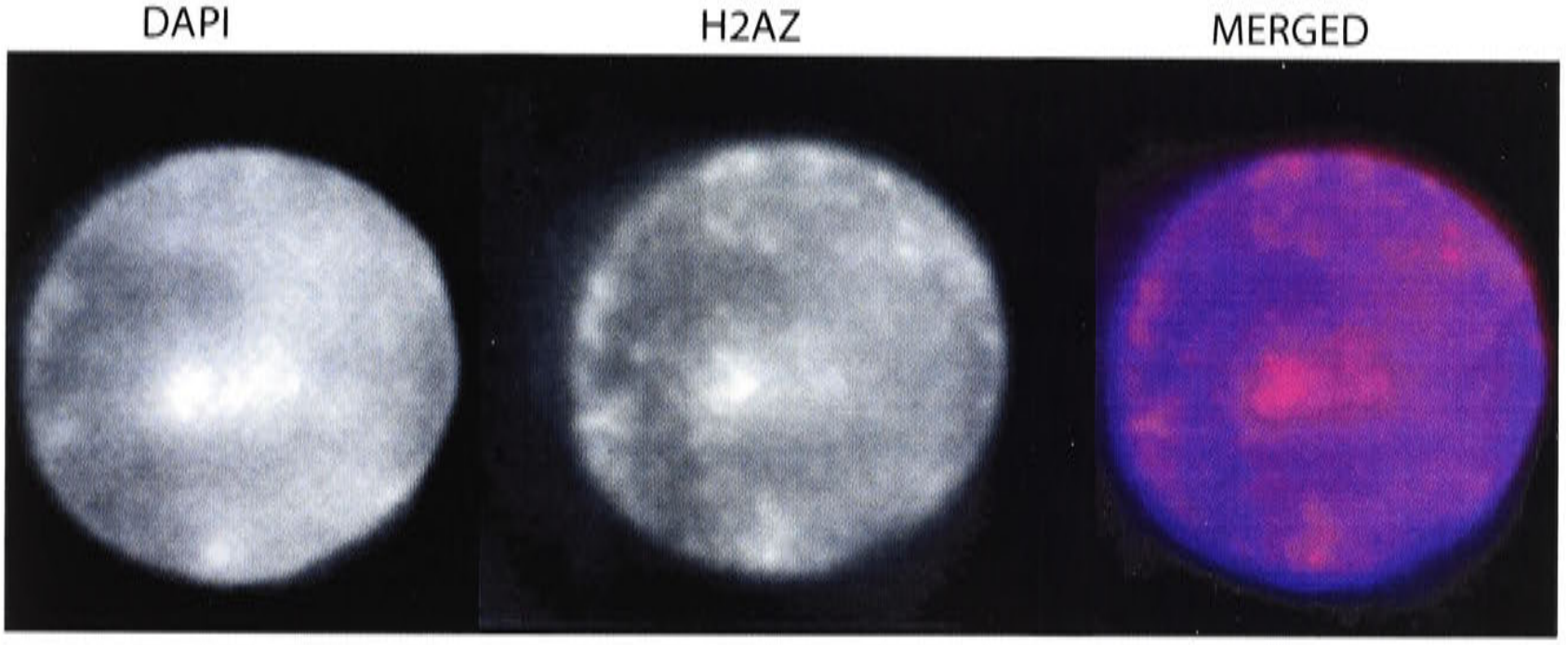


Figure 6.19: H2AZ in dunnart spermatid sections. In sections H2AZ localized to round spermatids, co-localizing with brighter DAPI regions.

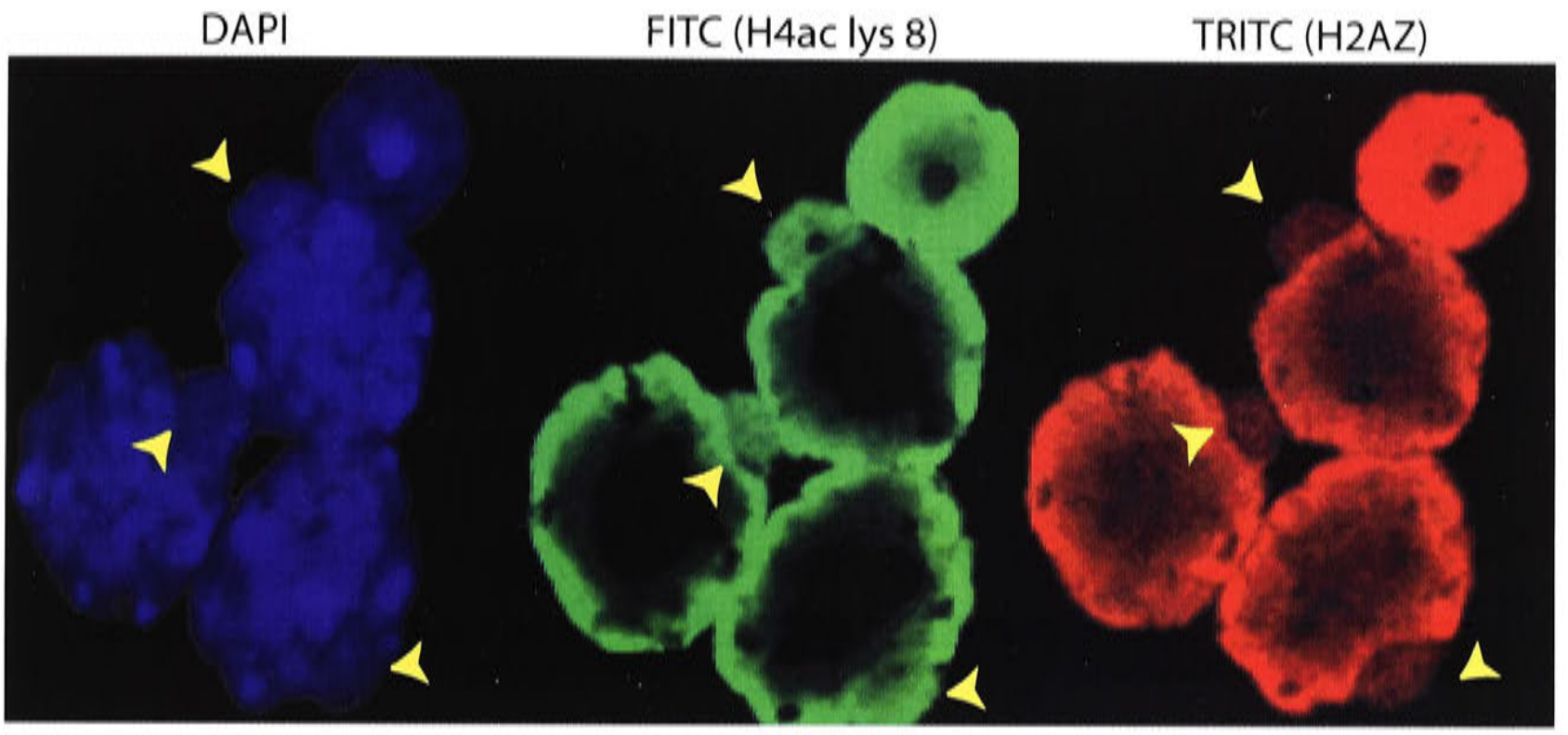


Figure 6.20: Confocal microscope picture of three mouse pachytene cells and a stage 8 round spermatid double stained for H4ac lys 8 (FITC, green) and H2AZ (TRITC, red). In all three pachytene cells the SV (➤) is stained for H4ac lys 8, but lacks H2AZ staining.

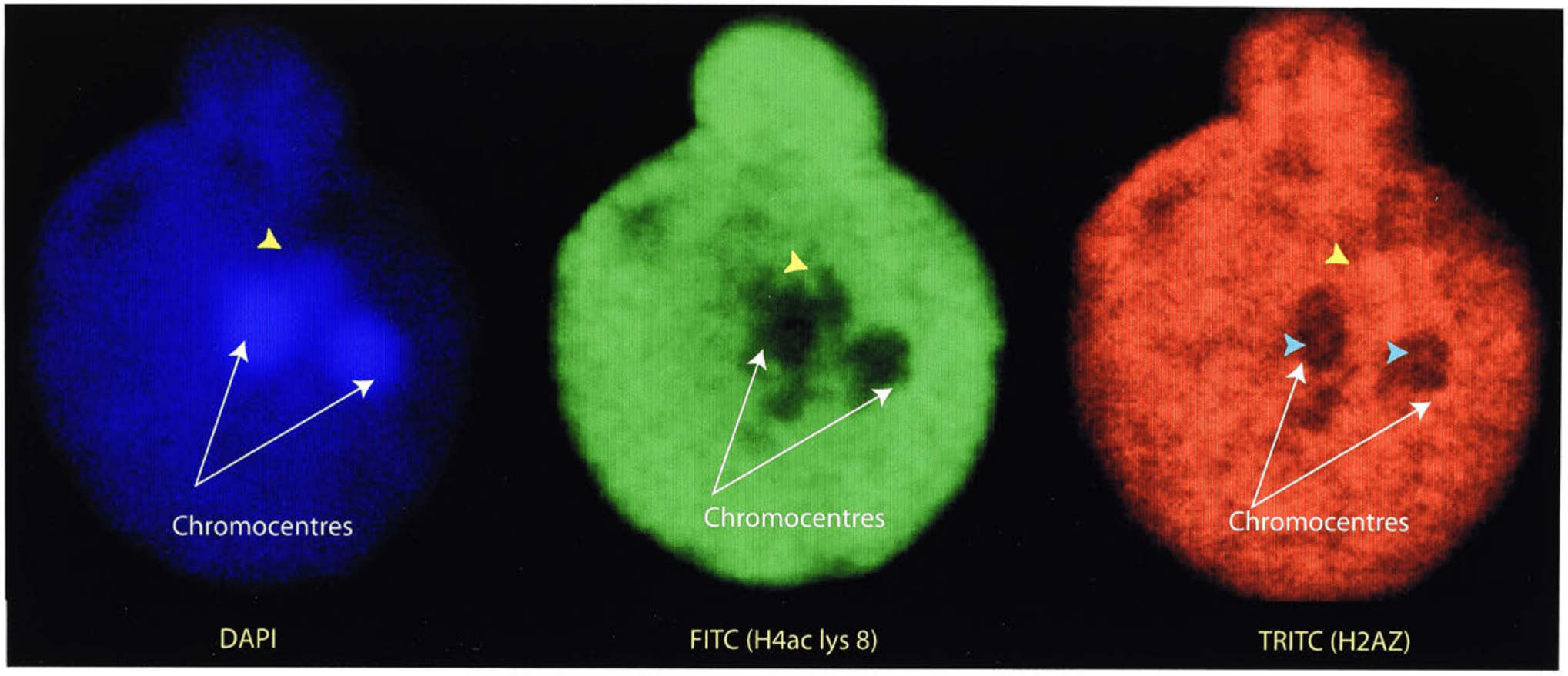


Figure 6.21: Confocal microscope image of a mouse st 4-6 round spermatid stained with antibody to acetylated histone (FITC, green) and H2AZ (TRITC, red). The shaded area next to the chromocentres lacks H4 acetylation (➤). This corresponding region is enriched in H2AZ. A small dot of H2AZ (➤) was observed in the middle of the chromocentres.

middle of the chromocentre in a round spermatid (figure 6.21). This dot was not seen in 2-dimensional microscopy as staining from the rest of the cell masked its presence.

A confocal microscopy image was taken of a mouse elongating spermatid st. 9-12 that showed two interesting features (figure 6.22). One was an odd distribution pattern of H4ac staining. This may be due to cell damage or may represent the replacement of histones with protamines. However, until more cells are studied this cannot be known for sure. The second interesting observation was H2AZ staining at the same focal plane as DAPI – bright centromeres at the centre, implying that centromeres are packaged with H2AZ in elongating spermatids.

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6.3 Discussion

It is therefore apparent that although the distribution of H2AZ and H4ac overlap, there are also some important differences. The distribution of mH2A was quite different from H2AZ or H4ac. Thus all three different modifications or variants have their own pattern of distribution and presumably roles within different stages of mouse spermatogenesis (figure 6.23).

6.3.1 Histone acetylation

6.3.1.1 Histone acetylation in somatic cells

Histone acetylation patterns on the inactive X chromosome were found to be conserved between eutherians and marsupials. In the hybrid mouse line, the inactive *M. caroli* X chromosome(s) was underacetylated. Similarly in tammar wallaby cells, the long euchromatic arm of one X chromosome was hypoacetylated. These results corroborate the observations of both Jeppesen (1993), and Wakefield (1997).

In contrast to both marsupials and eutherians, in cells from a platypus female there was no differential H4 acetylation of the X chromosomes. The NOR regions on both chromosome 6 were hyperacetylated in metaphase cells but not at interphase. Acetylation may reflect the activity of the ribosomal genes, however, this would be expected to occur during interphase. Alternatively, a strung – out, open chromatin configuration of the NOR region, which is made up of rDNA genes and repeats may inhibit proper condensation of this region. A third possibility is that during replication

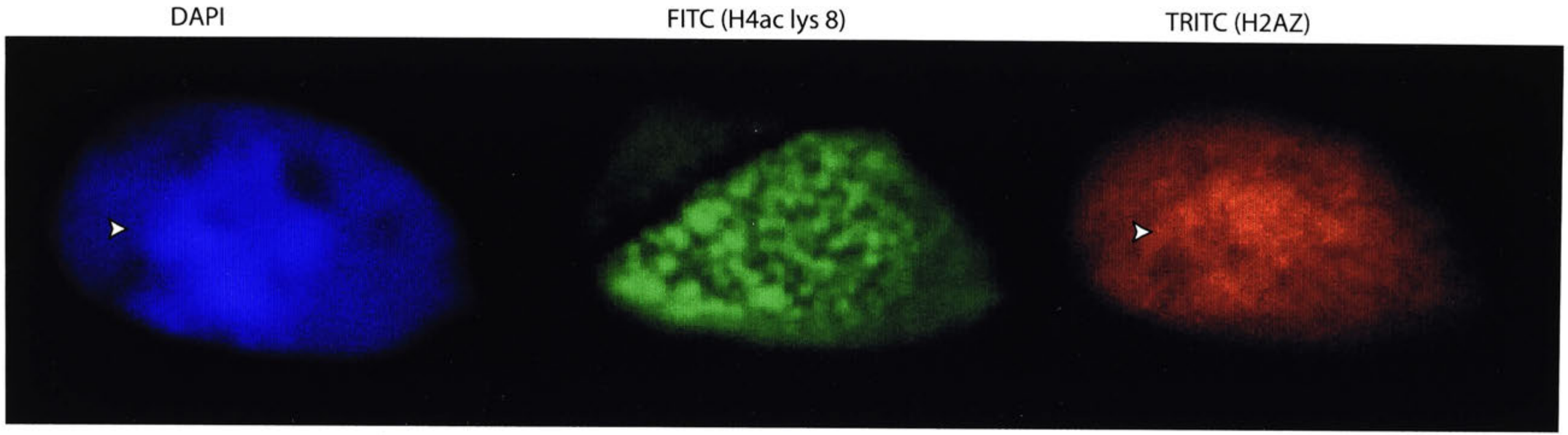

Figure 6.22: Confocal microscope image of a mouse stage 9-12 elongating spermatid. Immunostaining for H4 acetylation shows a strange pattern that may represent an ordered replacement of histones with protamines. Contradictory to the lack H2AZ staining of the chromocentre in round spermatids, H2AZ stains the chromocentre in elongating spermatids (▷). Centromeres can be observed in the cells via the bright dapi stained region.

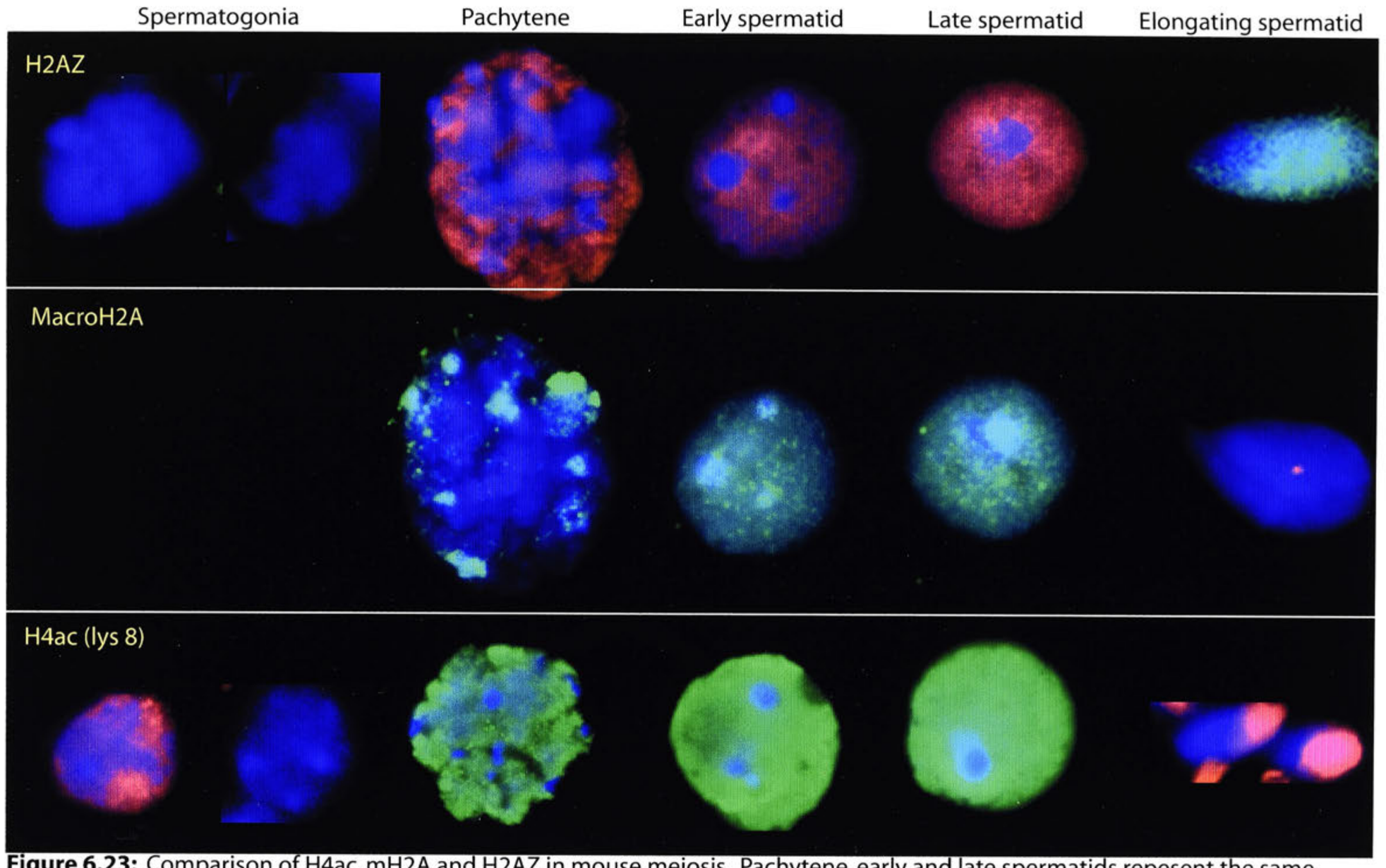

Figure 6.23: Comparison of H4ac, mH2A and H2AZ in mouse meiosis. Pachytene, early and late spermatids repesent the same cell stained with MacroH2A and H2AZ.

and division, the newly synthesized DNA is packaged with acetylated nucleosomes (known as deposition) that are then deacetylated (Sobel et al., 1995). This histone acetylation may not be involved in gene expression, but in forming the chromatin structure with the newly synthesized DNA. Therefore acetylation patterns may depend on the replication timing of particular regions of DNA. For example, in the field bean heterochromatin, euchromatin and the NOR regions have different acetylation patterns associated with packaging the newly synthesized DNA. Euchromatin is most strongly acetylated early in the replication process, whereas heterochromatin is strongly acetylated towards the end of replication, during mitosis and chromosome condensation (Jasencakova et al., 2000). In the platypus the NOR region is late replicating (Wrigley and Graves, 1988b), so the heavy acetylation pattern seen only on metaphase chromosomes may be due to the packaging of newly synthesized DNA with acetylated nucleosomes.

6.3.1.2 Histone acetylation in spermatogenesis

There is contradictory evidence concerning the histone acetylation state of mouse pachytene cells. Hazzouri (2000) claimed that pachytene cells are not acetylated, contradicting a report of acetylation in mouse pachytene cells (Armstrong et al., 1997). Approaches other than directly observing histone acetylation patterns via antibodies specific for the H4 acetyl isoforms, may help in resolving this argument. (Grimes and Henderson, 1983).

A high level of histone acetylation is consistent with high incorporation of [^{3}H]-uridine in pachytene and round spermatids (Kierszenbaum and Tres, 1974). It may make sense that spermatocytes and spermatids are transcriptionally active since many genes are transcribed in these cells , but are not translated until later. For example, there is the transcription of transitional proteins and protamines in round spermatids, but a delay in translation until the spermatids are elongating (st. 9-16) (reviewed by Kleene, 2001).

Also consistent with the idea of an open configuration of chromatin in pachytene is the finding that the testis specific histone 1, which favours open chromatin domains that require H4 acetylation, is found in high levels in pachytene cells (Khadake and Rao, 1995). Access to chromosomal DNA is very important for a number of meiotic functions such as pairing, recombination and double stranded beaks, all of

which occur in pachytene cells. An open chromatin state, and therefore histone acetylation, may be essential for these processes to occur.

The present findings of acetylated histones and high levels of H2AZ (which is associated with active DNA) in pachytene cells are also consistent with an active cell with open chromatin (Adam et al., 2001). All this evidence suggests that pachytene cells are actively transcribing cells with an open chromatin state, apart from the SV and centromeric heterochromatin.

Thus my results, as well as those of Armstrong, contradict the report of Hazzouri (2000), who observed no localization of antibodies to acetylated histones in pachytene cells, nor could demonstrate acetylated histones via western blotting of pachytene cells, (work not presented but referred to by (Hazzouri et al., 2000a), which would suggest a cell with little or no gene activity and a closed chromatin state.

It is possible that the contradictory results are due to differences between antibody staining in sections and surface spread preparations. In the present study very little histone acetylation was detected at pachytene in mouse testis sections, but in pachytene cells prepared by the surface spreading technique, H4ac lys 8 signal was detected throughout most of the cell nucleus. Similarly histone acetylation was detected inconsistently in round spermatids in sections, but was observed consistently in all round spermatids prepared by the surface spreading technique. In surface spreads (in the present and published work), mH2A was found to be concentrated at the chromocentre. Although my surface spread preparations demonstrated this, my sectioned preparations lacked mH2A at the chromocentre. Inconsistent labeling therefore seems to be a problem of accessibility of histone antibodies to sections.

The present results therefore confirm the majority view of histone acetylation and gene expression in pachytene cells that are actively transcribing and open to recombination and repair.

6.3.2 MacroH2A

6.3.2.1 MacroH2A in somatic cell X-inactivation

MacroH2A localizes to the inactive X chromosome in female eutherian somatic cells, but it is not essential for inactivation (section 1.6). The aim of this study was to observe if mH2A is also involved in marsupial X-inactivation.

No evidence was found for a specific mH2A body localizing to the inactive X chromosome in marsupials or monotremes. In both male and female marsupial

fibroblasts a macro chromatin body (MCB) was observed outside the nucleus but not on the inactive X chromosome. In platypus no MCB was observed within the cell in either males of females. However, in pachytene cells of marsupials a MCB was observed on the sex vesicle. Therefore the role of mH2A in maintaining X-inactivation is not a conserved feature of mammalian somatic cell X-inactivation.

Probably mH2A induces inactivation of the mouse X by contributing to heterochromatinization. The inactive X chromosome becomes heterochromatic and forms the sex chromatin body (Barr and Bertram, 1949). The observation that the inactive X chromosome of marsupials does not consistently form a heterochromatic sex chromatin body may be a direct result of the absence of mH2A localization to the inactive X chromosome. This may explain why mH2A did not localize to the inactive X chromosome in marsupial somatic cells but did localize to the heterochromatic SV in marsupial pachytene cells.

6.3.2.2 MacroH2A in spermatogenesis

Although, macroH2A evidently does not have a conserved role in mammalian somatic cell inactivation, it could have a conserved role in sex chromosome inactivation in mammalian meiotic cells. The finding of the present study that the SV contain mH2A in marsupials as well as eutherians suggests that its role in meiotic X inactivation predates eutherian/marsupial divergence of 130mya.

Inactivation of the SV at meiosis has been suggested to be an ancient system predating the evolution of somatic X-inactivation (Ayoub et al., 1997). Data from the present study lend support to the role of mH2A in inactivation of meiosis preceding its role in somatic cell X-inactivation (figure 6.24).

Xist RNA coating is required for localization of mH2A to the inactive X in mouse somatic cells, and *Xist* is expressed in pachytene cells in mouse male meiosis where there is also a MCB (Richler et al., 2000, Hoyer-Fender et al., 2000a, Ayoub et al., 1997). In the present study, the observation that mH2A coats the SV suggests that XIST may be expressed in marsupial meiosis. My data imply that generally the mechanisms involved in mammalian meiosis are extremely conserved, as little differences in acetylation or histone variants could be found between mouse and marsupials.

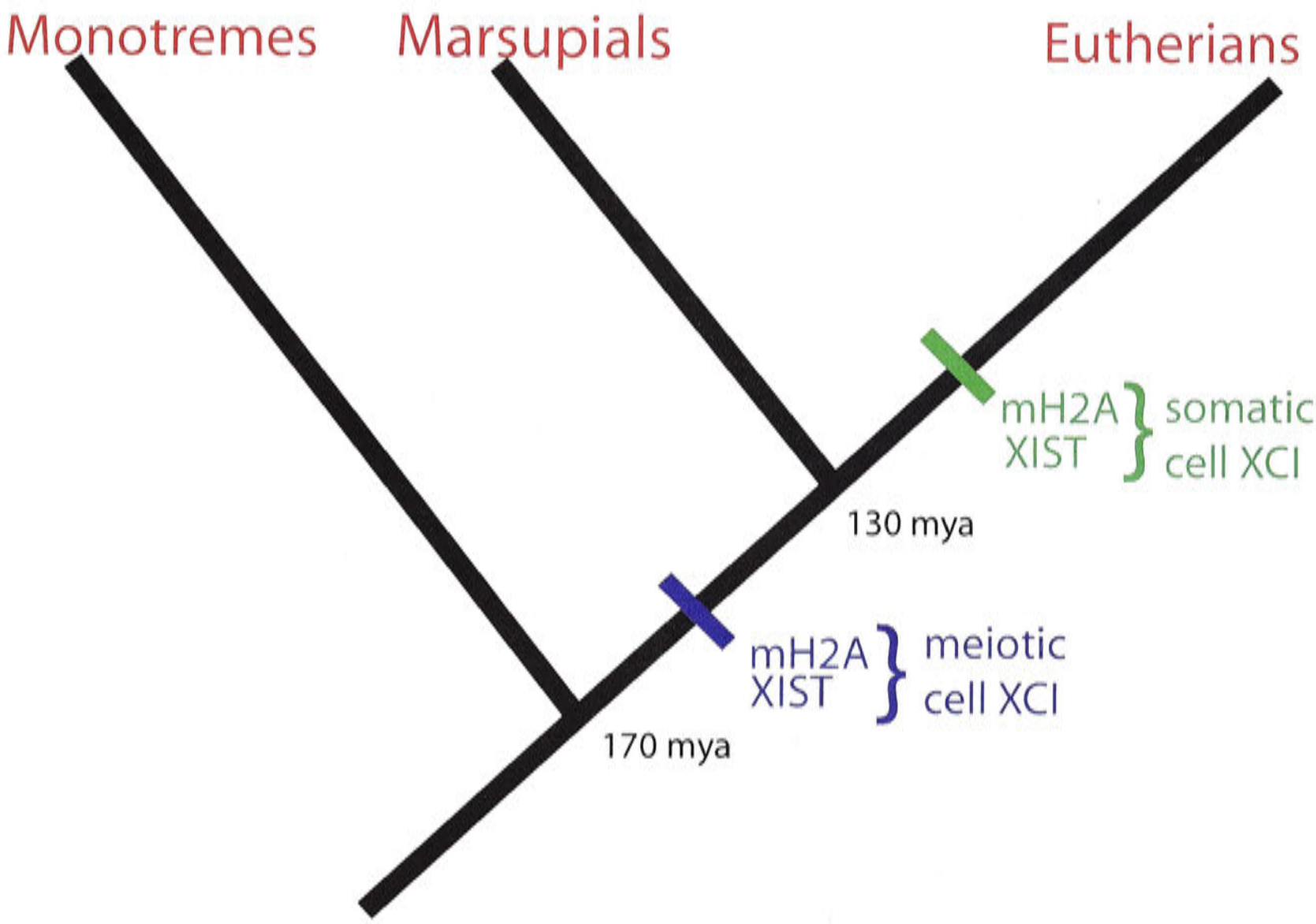

Figure 6.24: Evolution of macroH2A and XIST in meiotic and somatic cell X-inactivation. MacroH2A and XIST first evolved in meiotic X-inactivation after the divergence of Prototheria from Theria, but before the divergence of marsupials and eutherians. The role of mH2A in somatic cell X-inactivation evolved later, after the divergence of marsupials and eutherians.

6.3.3 H2AZ

H2AZ is the first histone variant found to be essential for development, so it is likely to play an important role in cell differentiation (Clarkson et al., 1999, Faast et al., 2001). I therefore studied the pattern of occurrence of H2AZ in mouse meiosis. Meiosis is an ideal system in which to study H2AZ expression and to try to determine its function. Spermatogonia go through several rounds of mitosis and then differentiate into Spermatogonia B, which become preleptotene cells and proceed through meiosis. Spermatocytes undergo replication, pairing, recombination and division; round spermatids undergo differentiation and transcription before their transition into spermatozoa, as well as the removal of histones and repackaging of DNA with protamines at stage 9-16 spermatids. I therefore hoped to confirm the role of H2AZ in chromatin organization and correlate its presence with a particular function in meiosis.

In this study, H2AZ was observed in mouse spermatocytes and spermatids but not in spermatogonia. Thus the presence of H2AZ seems to be correlated with activity. H2AZ was found to be highly expressed in round spermatids, which are known to have high levels of transcription, including the transcription of protamine genes whose translation is delayed until later on (reviewed by Kleene, 2001). H2AZ was also found at high levels in pachytene cells, which also have transcription (reviewed by Kleene, 2001), except for the heterochromatic SV, which was deficient in H2AZ.

My observation that H2AZ did not localize with any constitutive heterochromatin (such as the chromocentre) or facultative heterochromatin (eg. the inactive X) is consistent with its inhibition of heterochromatin formation (Fan et al., 2002). A manganese ion bound to H2AZ at His 112 at the surface of the nucleosome may act as a binding site for other proteins that regulate transcription in some way. In mouse round spermatids, active transcription for an enrichment of H2AZ was seen directly adjacent to the chromocentre providing? the one inconsistency to the correlation of H2AZ. This region directly adjacent to centromeres may be the nucleolus, which in yeast and metazoans is known to be enriched for H2AZ (Allis et al., 1982, De Leo et al., 1999). A proliferation marker (protein pKi-67) is found to hybridize to the centric and pericentric heterochromatin and also to a region adjacent to the chromocentre, which was attributed to the nucleolus (Traut et al., 2002).

Using 3-D confocal microscopy I also identified a small dot of H2AZ staining in the middle of the chromocentre of mouse spermatids. This suggests localization of

H2AZ to part of one or more centromeres within it. This could represent packaging of centromeres with H2AZ early in spermatogenesis prior to replacement with protamines. However, I was unable to determine what this dot may be or what function it may serve. It has been suggested that the bright DAPI region attributed in this study to the chromocentre, may, in fact, be the nucleolus (David Tremethick, personal communication) A similar structure observed in Sertoli cells is known to be the nucleolus. If this is this case then the small dot of H2AZ in the centre of the bright DAPI region may be the small region of active rDNA genes embedded within nucleolar heterochromatin.

During stages 9 to stages 11-12 histones undergo numerous modifications such as ubiquitination and acetylation. These modifications contribute to inhibiting nucleosome-nucleosome interactions and may also play important roles in nucleosome destabilization to allow histone replacement. Ubiquitination may also play a role in tagging dissociated histones for degradation. I found H2AZ in these stages, suggesting that H2AZ plays some role in histone replacement, perhaps in inducing nucleosome instability. Within the nucleus, the interactions between H2AZ and H2B are unchanged (when compared to interactions of H2A and H2B) and the interaction between the H2AZ-H2B dimer and the DNA is unchanged, but there is a change in the association between the (H2AZ-H2B) dimer and (H3-H4) tetramer because H2AZ nucleosome core particles have a reduced stability, and this destabilizes dimmer-tetramer interaction (Suto et al., 2000, Abbott et al., 2001). Therefore in co-operation with other modifications, H2AZ may contribute to the breakdown of the nucleosome as part of replacement with protamines.

Alternatively, the incorporation of H2AZ at these spermatid stages may produce a specific pattern of higher chromatin folding that is needed for histone replacement with protamines. As explained in section 1.3.2.1, phosphorylated protamines bind to the DNA and replace histones in an ordered process that allows a controlled condensation of the sperm nucleus. Binding with H2AZ positions nucleosomes in an ordered array (Fan et al., 2002), so it may produce a chromatin state that allows easy access and ordered incorporation of transitional proteins and protamines (figure 6.25).

My confocal microscopy observations of H2AZ incorporation at centromeres in elongating spermatids are the first indication that H2AZ may be associated with centromeric DNA. This may mean that H2AZ has an essential role in the replacement

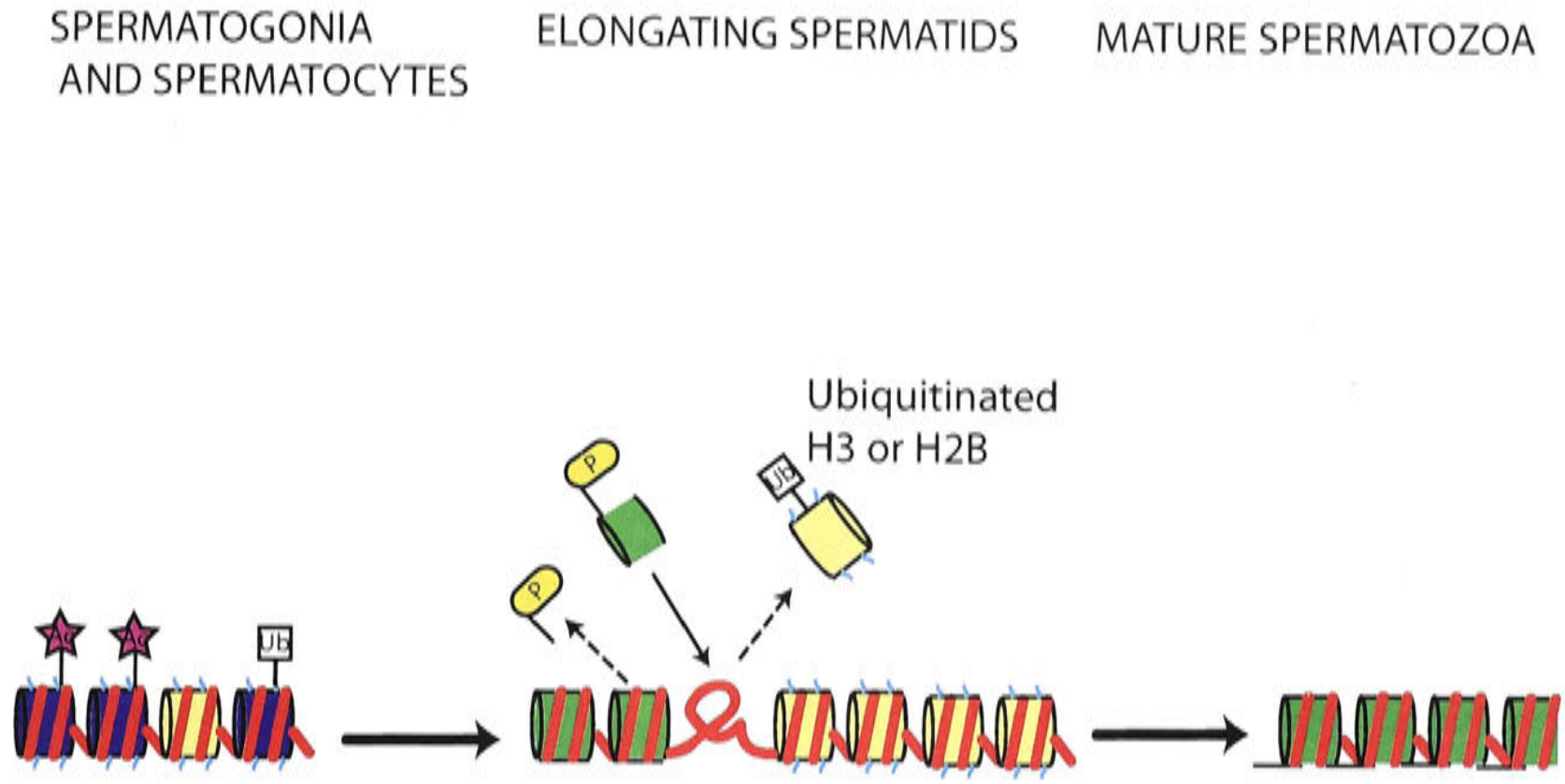

Figure 6.25: Chromatin remodeling during spermatogenesis.
H2AZ containing nucleosomes may play an important role in the transition of the nucleohistone to the nucleoprotamine complex. H2AZ may produce a chromatin state essential for the displacement of the nucleosome and the binding of firstly the transitional proteins and secondly the protamines. Ubiquitin may play an important role in the process or could be used as a tag for histone degradation.

of histones with protamines by opening up the centromeric DNA to allow integration of the protamines.

6.4 Summary

The concept of a "histone code" leads to the idea that different histone modifications and variants may affect each other within a nucleosome.

In this study I observed the relationship of three different histone forms through mouse meiosis. At the beginning of meiosis some spermatogonia have acetylated histones, but no mH2A or H2AZ. At the beginning of spermatocyte formation, histone acetylation was found to continue and H2AZ to appear, except at heterochromatic regions which are packaged with mH2A. As the early round spermatids matured, H4 acetylation, H2AZ and some mH2A were detected throughout the cell with mH2A concentrated at the chromocentre (figure 6.26 and 6.27). In elongating spermatids, mH2A was no longer present, but H2AZ, acetylated H4, acetylated H3, acetylated H2A, acetylated H2B, ubiquitinated H3, are all found (Hazzouri et al., 2000a). This high proportion of modifications and variants affects higher chromatin folding and nucleosome stability, allowing replacement of histones with protamines.

In this study, I examined the relationship between distribution patterns of different H2A variants and acetylated H4 during mouse and marsupial spermatogenesis. This revealed an inverse relationship between histone variants H2AZ and mH2A in which regions of H2AZ enrichment lack mH2A, and regions of mH2A enrichment lack H2AZ. In pachytene cells, the centromeric heterochromatin and the SV were enriched in mH2A but lacked H2AZ, and in round spermatids the chromocentres were enriched for mH2A but lacked H2AZ. A region directly adjacent to the chromocentre was enriched in H2AZ and lacked mH2A. This inverse relationship is consistent with an association of mH2A with heterochromatin, and a role of H2AZ in inhibiting the process of heterochromatinization (Fan et al., 2002).

Although acetylated histones and H2AZ have both been correlated with transcription, they have different patterns within meiosis, such as histone acetylation of the transcriptionally inactive SV, and H2AZ enrichment at centromeric heterochromatin or the nucleolus. The roles of H4 acetylation and H2AZ may therefore be different in different cell types. Acetylation of H4 on the transcriptionally inactive SV may produce

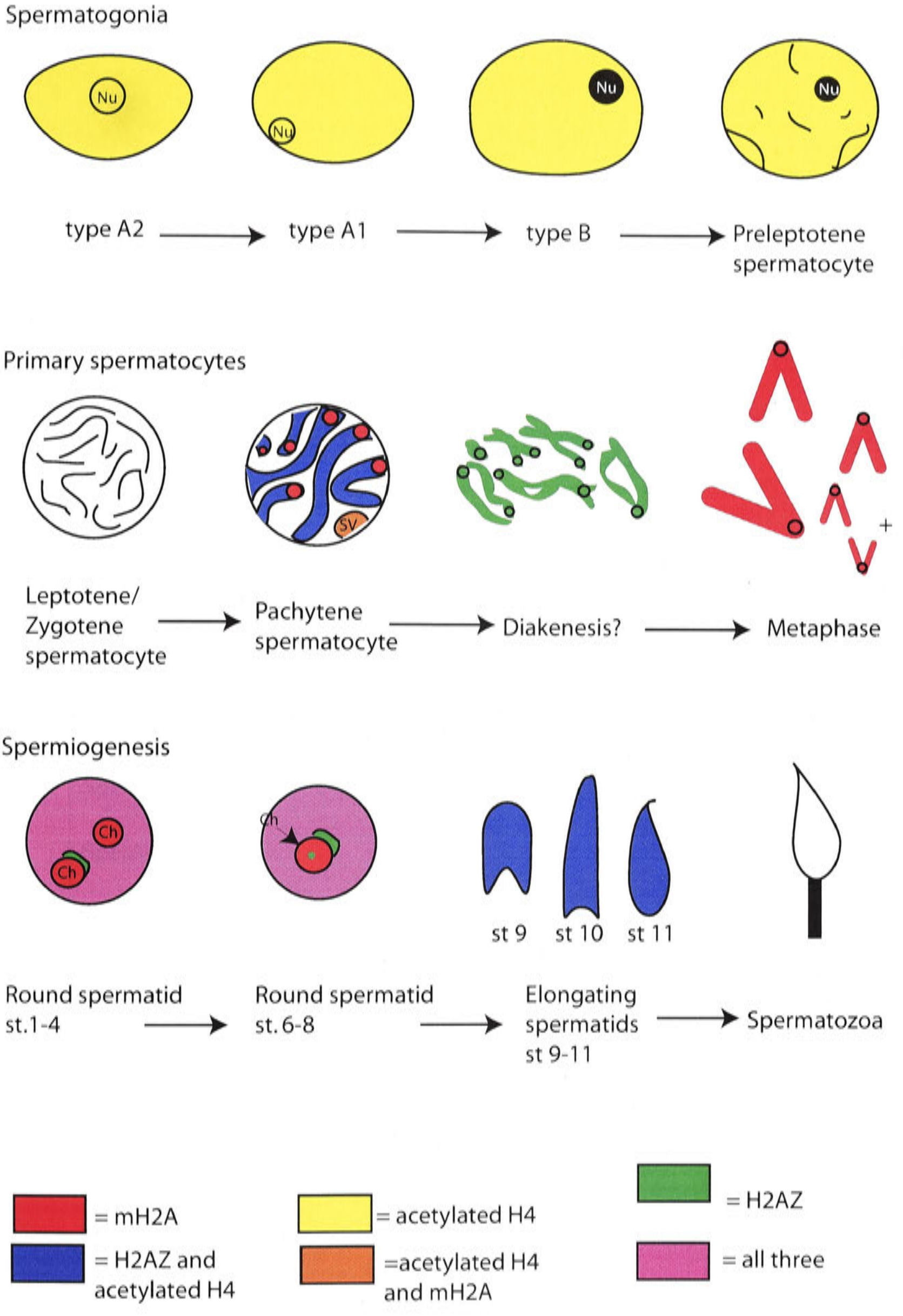

Figure 6.26: Histone patterns during mouse spermatogenesis. Different cell types show different patterns of histone variants and modifications. + Result taken from Hoyer-Fender *et al*, 2000. Ch = chromocentre; ○ = centromeres; Nu = Nucleolus.

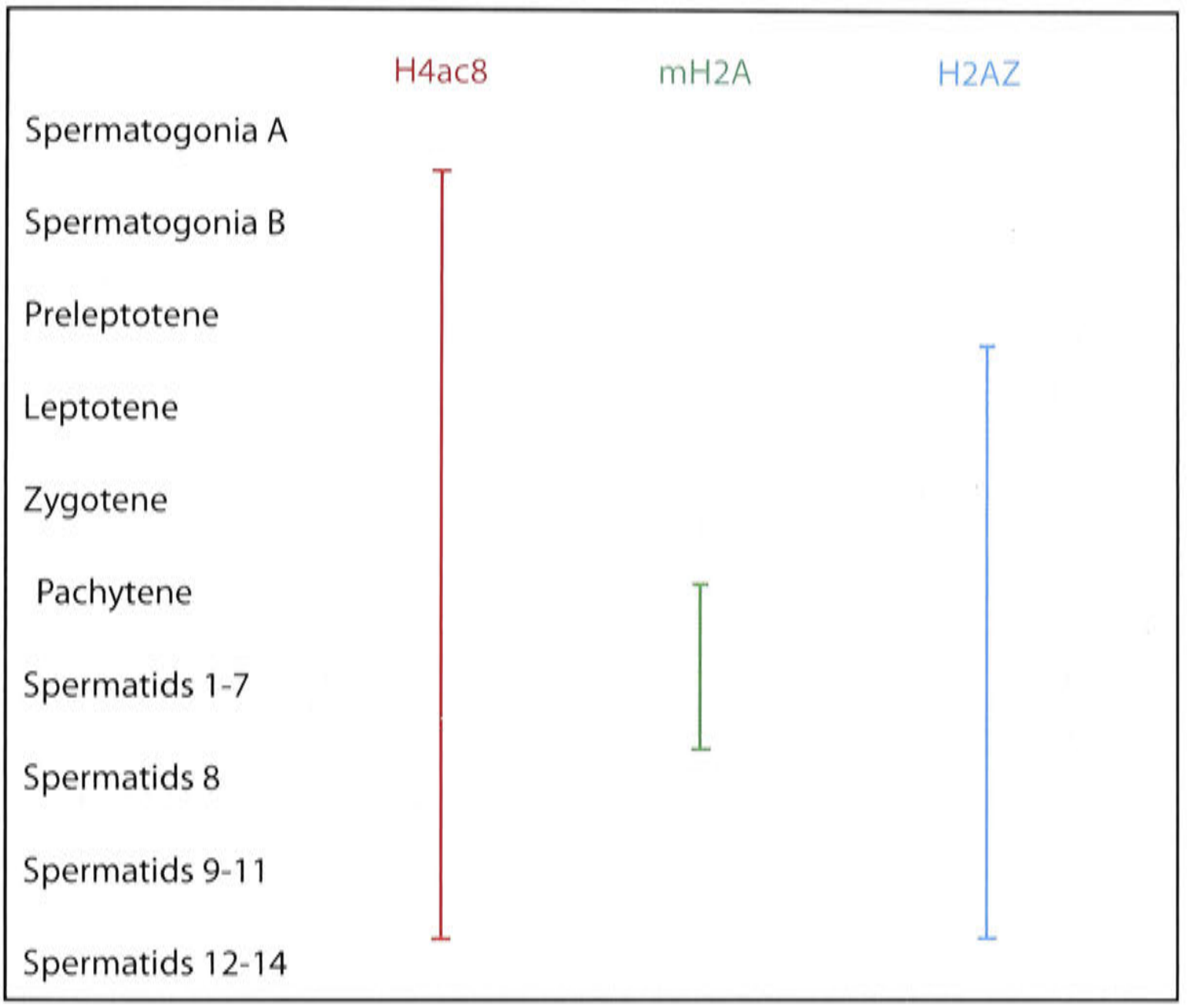

Figure 6.27: My observation of histone patterns of H4ac lys 8, mH2A and H2AZ in mouse spermatogenesis.

a chromatin state susceptible to integrating XY specific proteins. Acetylation of lysine 12 is known to help promote heterochromatinization in *Drosophila*, so histone acetylation of the SV may actually help in heterochromatinizing the X chromosome by acting as binding sites to specific SV specific proteins.

H2AZ also seems to have two different functions, as it may promote transcription yet also be involved with transcriptionally inactive DNA such as pericentromeric heterochromatin. H2AZ is known to act on higher order chromatin folding, so H2AZ containing nucleosomes may act as a intermediary between active and inactive chromatin by producing a chromatin state open for either activation or inactivation (figure 6.28) (Fan et al., 2002). H2AZ may make the DNA accessible for acetylases, ubquitinases or transcription factors (eg. Pol II) enhancing transcription levels. In contrast methylases, and heterochromatic proteins may be able to attach to the DNA to cause inactivation.

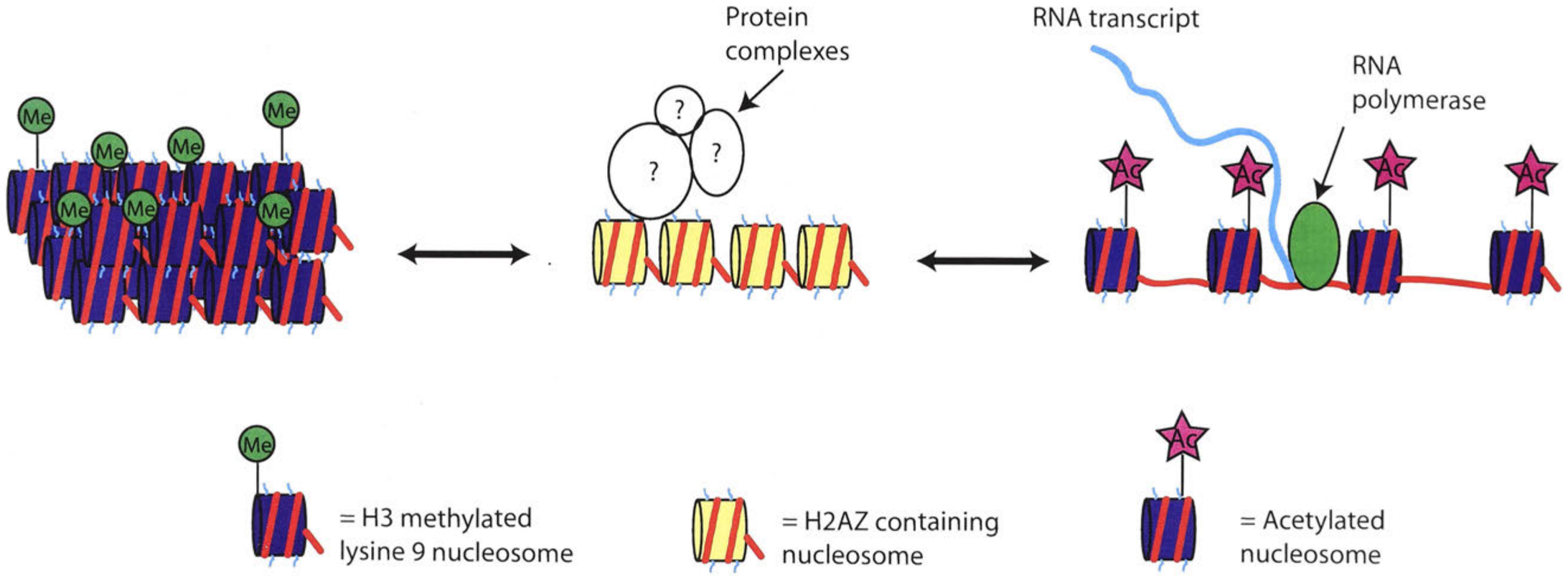

Figure 6.28: H2AZ containing nucleosomes may promote a chromatin state intermediate between heterochromatin and euchromatin that is open for protein binding. Different proteins (eg. acetylases, methyltransferases) may bind to the metal ion in H2AZ and influence chromatin structure.

CHAPTER 7: CONSERVATION OF CHROMOSOME AND CHROMATIN ORGANIZATION DURING MAMMALIAN SPERMATOGENESIS

The aim of this study was to compare the changes in nuclear organization through mammalian spermatogenesis in marsupials and eutherians in order to deduce the role of chromosome and chromatin organization in mammals. In previous work I demonstrated that chromosomes have a non-random arrangement in dunnart sperm. During my PhD project I studied chromosome arrangement in vertebrate sperm in more detail to establish its generality and deduce its function, then I traced how this arrangement was set up in sperm, and studied chromatin arrangement and binding with modified and variant histones. This study has established four observations about chromosome arrangement and chromatin structure in mammalian meiosis and sperm:

1. A non-random chromosome arrangement is a general feature of mammalian sperm.
2. The non-random arrangement of chromosomes in mammalian sperm does not occur in chicken sperm, suggesting a mammalian specific function for the arrangement.
3. The X chromosome has a conserved position at the anterior of mammalian sperm that may have, or may have had a role in the ancestral mammalian X-inactivation system.
4. There is an inverse relationship between distribution of two histone variants, mH2A and H2AZ in mouse, suggesting that H2AZ may play an important role in the nucleohistone to nucleoprotamine process.

7.1 Conserved chromosome position in mammalian, but not chicken sperm

A non-random arrangement of chromosomes was demonstrated in wombat as

well as dunnart and platypus sperm, but not in chicken sperm. This, with the evidence of non-random arrangement of chromosomes in eutherian sperm, suggests that a non random chromosome arrangement is a conserved feature of mammalian sperm. Furthermore, I demonstrated that in wombats and dunnarts, which have nearly identical karyotypes, the chromosome arrangement is the same, implying that it has been conserved for at least the 50 myrs since these species diverged. Conservation of a chromosome arrangement over such a long period suggests that it plays some important role. This function could be structural, important for the integrity of the cell, or functional, perhaps setting up transcriptional domains in the zygote.

7.1.1 Structural integrity of sperm

The chromosome arrangement may be important for mechanical reasons. The non-random arrangement of chromosomes may be critical for the mechanical stability of the sperm. The non-random chromosome arrangement may allow a stable transmission of DNA, minimizing the amount of DNA damage. This is very different from the original "bowl of spaghetti model", which depicts DNA as randomly and haphazardly packaged within the cell. DNA packaged in such a way would be susceptible to damage and decrease the success rate of offspring. However, the random position of chromosomes in chicken sperm suggests that the order is not developed for mechanical stability, although it has been suggested that the central location of microchromosomes in interphase cells protects these gene-rich elements from damage.

Alternatively the arrangement in sperm may be the consequence of chromosome distribution from a non-random meiotic process. During spermatogenesis, many changes occur to the spermatid DNA, including condensation and replacement of histones with protamines. The ordered arrangement of chromosomes observed in mammalian sperm may be a mechanical by-product of the histone-protamine replacement process. As previously described (section 1.3.2.1), the histone replacement process in spermatogenic stages 9-16 is highly ordered, with the specific positioning of protamines along the DNA. The process also occurs in a way that allows an ordered packaging of the DNA into the sperm nucleus. The non-random arrangement of chromosomes may be an extension of this where the ordered replacement of histones and packaging of the DNA produces a specific chromosomal order that has a structural role but is not significant for gene activation.

7.1.2 Random chromosome arrangement in chicken sperm

The observations that chromosomes are highly organized in mammal sperm but not bird sperm imply that an ordered arrangement evolved early in mammal evolution 170-310 mya. This occurred in the absence of a change in sperm morphology and development, which is very similar in monotremes and birds.

This observation rules out the possibility of an essential structural role for a fixed chromosome arrangement in mammal sperm. The non-random arrangement observed in mammalian sperm, therefore suggests that a non-random arrangement evolved to serve a mammalian specific function.

The defined arrangement of chromosomes in the sperm nucleus may have evolved as a component of a mammal-specific regulatory mechanism. In particular, the anterior position of the X chromosome in monotremes contrasts strongly with the random position in chicken sperm of the Z chromosome. Since the time that the X took up its anterior position in sperm coincided with the evolution of X chromosome inactivation in mammals, perhaps the evolution of paternal X chromosome inactivation (thought to have been the ancestral form, (Cooper, 1971) represents such a mammal-specific regulatory system.

7.1.3 Transmission of functional chromosome domains between generations

Alternatively, the non-random arrangement of chromosomes in sperm may be critical for gene expression, either in the spermatogonia and spermatocyte or, more probably for setting up chromosome domains within the zygote. It is clear that chromosome arrangement in the zygote is highly non-random. Although there is no direct evidence for fixed position of chromosomes in the zygote, the paternal and maternal chromosomes are kept separate up until the four cell stage (reviewed by Haaf, 2001). The apparent non-random distribution of the paternal and maternal genomes may predispose alleles to imprinting by changes of the sperm DNA, which would allow recognition and separation from the maternal genome.

There is now clear evidence of specific non-random radial arrangements of chromosomes in adult somatic mammalian cells (Cremer et al., 2001). The position of a chromosome within the cell nucleus is directly correlated with its gene content and therefore transcriptional activity, with inactive chromosomes at the periphery and active

chromosomes towards the interior (Croft et al, 1999; Boyle et al, 2001). The position of chromosomes in the zygote may therefore be critical in establishing patterns of activity. The position of chromosomes in the sperm may predetermine the pattern in the pronucleus and in the zygote nucleus. For example, chromosomes found at the anterior of the spermhead may be moved to the periphery of the zygote nucleus, setting them up for inactivation. Conversely, chromosomes found at the anterior of the sperm nucleus may take up an internal position, setting up an active transcriptional pattern. The medial position of the microchromosomes in chicken sperm may predetermine their position in the interior of the zygote nucleus, setting up the active transcription of the microchromosomes. This internal positioning of the microchromosomes, when established, will then be transmitted to all cell descendents.

If chromosome position is important for function, the abnormal positioning of a chromosome may produce abnormal gene function, such as the abnormal position of the X chromosome in epileptic foci (Borden and Manuelidis, 1988).

It was observed that some variation occurred in chromosome position. For example, chromosome 2, which was normally found towards the posterior of wombat sperm, occasionally produced signals in the medial region of the spermhead. This may have been an artefact of analysis in two-dimensions, but could reveal abnormal chromosome positions in a minority of sperm. Sperm that carry an abnormal arrangement, as well as those that carry an abnormal chromosome complement, may not produce a normal zygote.

The position of a chromosome in relation to other chromosomes was not obviously fixed in pachytene cells. However, it was evident that the fixing procedure used destroyed the 3-dimensional positioning of chromosomes and observation in 2-dimensions did not allow an accurate interpretation of chromosome organization in pachytene cells. Therefore, it was not possible to deduce how and when the chromosome arrangement observed in sperm was set up.

7.1.4 Chromosome position and aneuploidy

Certain variants of chromosome number and rearrangements are much more common than would be expected by chance. For instance, most aneuplodies involve the sex chromosomes. The prevalence of XXY, XO and XYY may simply reflect the generally high viability of these genotypes. However, they could also result from the

apical position of the X chromosome in human sperm that may render it especially vulnerable to loss, as it is the first chromosome to enter the egg. This may be exaggerated during intracytoplasmic sperm injection (ICSI). In normal fertilization the acrosome breaks down totally and decondensation of the sperm nucleus happens all at once. However, in ICSI the DNA at the anterior of the sperm nucleus undergoes delayed decondensation due to the presence of the acrosome (Terada et al., 2000). Preferential loss of the X could result from the retention of the acrosomal cap after injection, which delays replication of the DNA at the apical region of the spermhead, where the X chromosome lies, leading to its lagging and loss (Luetjens et al., 1999, Terada et al., 2000).

Non-random chromosome rearrangements, which can cause developmental abnormalities or cancer, could also be the result of non-random chromosome positions in germ cells or somatic cells. For example, the high rate of t(9;22) leukemias in the human population can be explained by the close association of chromosomes 9 and 22 within the cell nucleus (section 1.2.2.2). This is a direct example of the position of a chromosome producing an abnormal chromosome complement that affects cell function eg. leukaemia.

7.2 Position of the X chromosome in sperm and establishment of paternal X-inactivation

If the evolution of mammalian XCI is proposed to have led to the evolution of an ordered chromosome arrangement in sperm, it is important to establish homology of the X between Prototheria and Theria, and the occurrence of XCI in monotremes as well as marsupials and eutherians.

7.2.1 The conserved mammalian X chromosome

In this study DNA from the flow-sorted platypus X chromosome was hybridized, across species, to human metaphase spreads. Its hybridization to the long arm and pericentric region of the X confirms the conclusion of gene mapping studies that a large region of the X has been conserved in all mammals. Although it was not possible to determine if the conserved sequences were unique or repetitive sequences,

the similar pattern of hybridization produced by DNA from the whole X and Xp suggest that the signal was due to repetitive sequences spread throughout this region. My observation that the strongest region of hybridization with the platypus X chromosome was at human Xq13-Xq21 (the site of the X control element) suggested that the signal represents a conserved mammalian repeat that acts as 'booster' elements along the mammalian X chromosome, helping to propagate/transmit the X-inactivation signal along the length of the X.

7.2.2 The evolution of the mammalian X-inactivation system

The evolution of the mammalian X-inactivation system can be deduced by comparing X-inactivation between the three mammalian groups. In marsupials there is late replication and underacetylation of the inactive paternal X chromosome, but no methylation of CpG islands and, as yet, little evidence of *XIST/TSIX*. Eutherians have late replication; methylation of CpG islands 5' to X-linked genes, *XIST/TSIX* expression, methylation of H3 lysine 9 and localization of macroH2A. In monotremes which diverged from Therians 170mya, there is contradictory evidence for X-inactivation from replication patterns, asynchronous replication of the short arm of one of the X chromosomes was observed, yet the short arm is paired by the element E2 and should need no dosage compensation (Wrigley and Graves, 1988b).

MacroH2A localizes to the inactive eutherian X chromosome after expression of *XIST* begins (Mermoud et al., 1999). It was not known if mH2A localized to the inactive X chromosome in marsupials. In this study I used antibody to mH2A to determine if mH2A also localized to the marsupial and monotreme inactive X, and was therefore a conserved feature of mammalian X-inactivation. No mH2A localization (ie. MCB) was observed within the marsupial cell nucleus that could be attributed to the inactive X, but a signal outside the cell nucleus was observed in female and male marsupial fibroblasts. This was attributed to mH2A localization at the centrosome. In mouse embryonic and somatic cells, mH2A also localizes to the centrosome, presumably the site at which mH2A is degraded (Chadwick and Willard, 2002, Mermoud et al., 2001). In monotremes there was faint staining of mH2A throughout the cell nucleus, but no MCB was ever observed.

The non-localization of mH2A to the inactive marsupial X chromosome may be relevant to the search for XIST in marsupials; since *Xist* must be transcribed and present

on the inactive X chromosome for mH2A to localize to the inactive X chromosome (Csankovszki et al., 1999). Certain regions of the *Xist* RNA are essential for recognition and binding of mH2A to the inactive X chromosome (Gilbert et al., 2000). The absence of a MCB from the marsupial inactive X may reflect the absence of XIST from the inactive marsupial X chromosome. If this is so, X-inactivation in marsupials may be very different from that in eutherians. This course of evolution of X-inactivation is represented in figure 7.1.

7.2.3 Functional significance of the X chromosome position

My work has demonstrated clearly that the X chromosome is located at the apex of the fibrillar spermhead in platypus. In marsupials, the X chromosome is positioned in the medial region of the spermhead that turns on a 90° angle from the tail to produce a T-shape at fertilization. The X chromosome lies in a medial position in marsupial sperm at the point of first contact with the egg. Published work has demonstrated that the X chromosome is non-randomly positioned at the apical region of the human spermhead. Thus, in all three mammalian groups the X chromosome would be the first chromosome of the paternal genome to enter the egg. This position may be critical in establishing a specific chromosomal domain within the zygote that is responsible for paternal X-inactivation.

This hypothesis can account for the apparent absence of nuclear organization in bird sperm, where there is no Z-inactivation and the chicken Z chromosome has a random position in sperm. The absence of a defined arrangement of chromosomes in chicken sperm suggests that selection for an ancestral mammal specific function imposed a non-random arrangement. In a common mammalian ancestor the X chromosome inactivation system may have initially depended, for inactivation, on the position of the X chromosome in the zygote, set up by its position in sperm. An imprint or the peripheral position of the paternal X chromosome within the zygote may have marked the paternal X chromosome for inactivation. Vestiges of the ancestral mammalian X-chromosome inactivation system may still be used in monotremes and marsupials, but not eutherians.

Eutherian X-inactivation followed a different evolutionary path after the divergence from marsupials, with the development of more elaborate maintenance systems such as methylation of CpG islands 5' from X-lined genes, *XIST/TSIX*

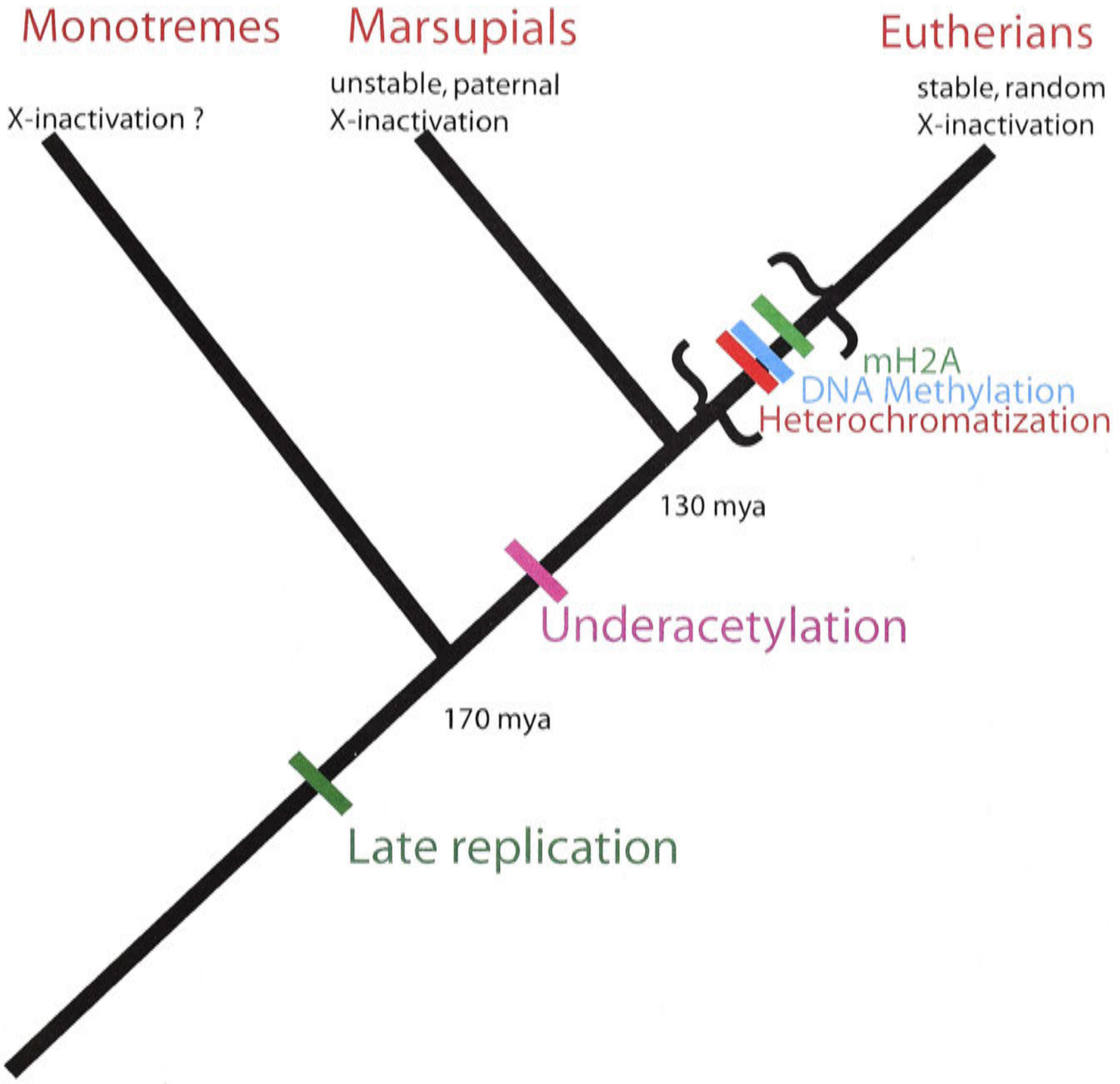

Figure 7.1: Evolution of the mammalian X-inactivation mechanisms. Eutherians developed additional mechanisms that allowed a more stable X-inactivation system and perhaps randomness.

expression and mH2A localization, which produce a more stable X-inactivation system. These evolutionary changes in eutherian X-inactivation may have made the ancestral X-inactivation system obsolete in eutherians, as X-inactivation is no longer dependent on X chromosome position, but expression of *XIST/TSIX*.

7.2.4 Models for marsupial paternal X-inactivation

In marsupials, X-inactivation is always paternal. This was the first example of genomic imprinting in a mammal. The apical position of the mammalian X chromosome in sperm correlates with imprinted paternal X-inactivation. The imprint may work in one of two ways,

1) The apical position of the X chromosome is associated with differential packaging with modified or variant histones
2) The apical position of the X chromosome delivers it to the periphery of the zygote nucleus, into a compartment which represses transcription

The first possibility is very plausible given that some of the DNA in sperm is packaged with histones rather than protamines. In sperm of the marsupial *Sminthopsis crassicaudata*, histones localize to the apical region of the spermhead and run down its dorsal surface adjacent to the acrosome. The X chromosome may associate with these histones, predisposing it to inactivation (Soon et al., 1997). The paternal X chromosome may enter the egg already packaged with histones primed for inactivation (methylated at H3 lysine 9, and/or hypoacetylated). During repackaging of the paternal pronuclei with histones from the egg, the histones packaged in the sperm DNA remain, and mark the X chromosome for inactivation. The packaging of histones also does not necessarily have to be a chromosome - wide characteristic, but could involve key imprints at locations along the X chromosome (perhaps the booster elements revealed by cross-species painting) that would promote the inactivation of the paternal X chromosome.

There is some evidence that such associations mark genes for early activity. For instance, the histones that bind 5' of the γ-globin gene may be acetylated, priming the gene for early expression in the zygote, whereas the promoter of genes expressed in adult cells may be bound with hypoacetylated histones, inhibiting early expression.

Similarly, the 15% of histones present in human sperm may be placed at strategic regions of the X, marking them for activation or inactivation once they enter the zygote. Histones may be bound to promoter regions of paternal X-linked genes, predisposing these genes for X-inactivation.

Alternatively, the peripheral position taken up by the paternal X chromosome in the zygote may influence its activity. There is a high correlation between chromosome position and activity in human cells, where active chromosomes are found towards the interior of the cell nucleus, and inactive chromosomes are towards the periphery . The peripheral position of the paternal X chromosome may influence its activity within the zygote, promoting paternal X-inactivation. This could occur if the X were isolated in a nuclear compartment that excluded transcription factors, or were rich in histone modifications or variants that repress genetic activity.

7.3 The mammalian sex vesicle and X-inactivation in mammalian meiosis

The structure and organization of the sex vesicle (SV) was studied in mouse and marsupials using chromosome painting, antibodies to modified and variant histones and electron microscopy. Chromosome painting revealed a balloon shaped structure familiar for all mammalian SVs. However, no pairing was observed between the X and Y, either by staining the synaptonemal complex, or by electron microscopy. SV structure in marsupials was independent of the relative orientation of the X and Y chromosomes at meiosis, confirming that no pairing occurs between the marsupial X and Y chromosomes and SV structure depends on telomeric binding to the nuclear membrane or dense plate. This leads me to speculate that X-Y pairing may not be essential for SV formation in eutherians either.

In order to understand how histone modifications and variants may influence X-inactivation at meiosis, antibodies to acetylated histones and antibodies to macroH2A and H2AZ were used to compare histone distribution in mouse and marsupial meiotic cells.

An antibody to acetylated histone H4 lysine 8 was used to confirm the results of Armstrong (1997) that histone 4 of the inactive sex vesicle in mouse pachytene cells is acetylated. This characteristic is also conserved in marsupials, in which acetylation of H4 was observed throughout the whole marsupial pachytene cell, including the

marsupial sex vesicle. .Meiotic specific proteins may bind to the inactive sex vesicle in mammals, inactivating the X and Y. Histone acetylation of the sex vesicle may play a role in acting as a binding site to proteins containing bromodomains that recognize the acetylated sex vesicle.

It was observed that no macro chromatin body (MCB) was observed on the marsupial inactive X chromosome in somatic cells in the present study, but a clear MCB was observed on the sex vesicle in marsupials as well as eutherians. The localization of the MCB, therefore, plays a conserved role in either sex vesicle formation, inactivation, or both, but not in somatic X-inactivation. Thus, mH2A seems to have a conserved role in X-inactivation in meiosis, but not in female mammalian somatic cell X-inactivation.

The absence of a MCB in female somatic cells in marsupials may relate to the absence of sex chromatin. The inactive X chromosome in somatic cells in mouse and humans forms a heterochromatic Barr body at the periphery of the nucleus that localizes with a MCB. However, in marsupial somatic cells, the X chromosome does not form a heterochromatic body at the periphery of the nucleus (McKay et al., 1987), and therefore, mH2A seems to play a direct role in heterochromatinization.

A newly characterized histone variant, H2AZ, was studied in meiotic cells of both marsupials and mice . Due to incorporation of the antibody in sectioned marsupial testis, little insight was gained of this variant in marsupials. However, through surface spreading techniques H2AZ was observed in mouse spermatogenic cells. H2AZ was demonstrated to bind throughout the pachytene cell nucleus, but was absent from centromeric heterochromatin and the sex vesicle. This finding is consistent with the proposal that H2AZ inhibits higher chromatin folding (Fan et al., 2002, Rangasamy et al., 2003). H2AZ was also present in round spermatids of both mouse and marsupial, which are actively transcribing cells, confirming previous results suggesting H2AZ may play a role in gene transcription.

7.5 Conclusion

I have studied chromosome and chromatin organization at many stages of meiosis and spermatogenesis. One common factor that emerged from the results of all these experiments was the ordered nature of processes and functions, from the pattern of histone distribution in meiosis to the tightly organized chromosomes in sperm. My

observation that the arrangement of chromosomes in sperm has been conserved over 170 million years and the association of histone 4 acetylation with X-inactivation for at least 130mya, suggests that arrangement and associations are highly conserved in mammals and have specific and significant functions.

The development of the zygote is a complex process, involving different levels of control of gene activity. Specific genes must be activated at specific times, and many RNA and protein relationships need to occur for the successful development and differentiation of the zygote. Two processes that must occur early on are the decondensation and repackaging of sperm DNA with histones, and the integration of the maternal and paternal pronuclei. Specific packaging of histones at critical regions in sperm DNA may act as initiating sites in establishing the nucleosome positions within the recently decondensed sperm chromatin (figure 7.2). These histones may also play an important role in establishing the pattern of early gene transcription within the zygote.

The finding that mH2A does not localize to the inactive X chromosome in marsupial somatic cells sheds new light on the evolution of the mammalian X-inactivation system. The X-inactivation system of the common ancestor of marsupials and eutherians included at least late replication and hypoacetylation. After the divergence of eutherians from marsupials, new mechanisms evolved that are not present in marsupials, producing a more stable X-inactivation system. These eutherian-specific mechanisms include DNA methylation at CpG islands 5' from X-linked housekeeping genes, *XIST* expression, heterochromatinization, and now, macroH2A localization, all of which provide a stable inactive state.

My PhD project has therefore contributed to the understanding of chromosome arrangement and chromatin organization during spermatogenesis in marsupials and its functional significance, particularly in X chromosome inactivation. I conclude that mammalian meiosis is an extremely conserved and ordered process that produces a specific chromosome and chromatin organization essential for setting up domains that control development of the zygote.

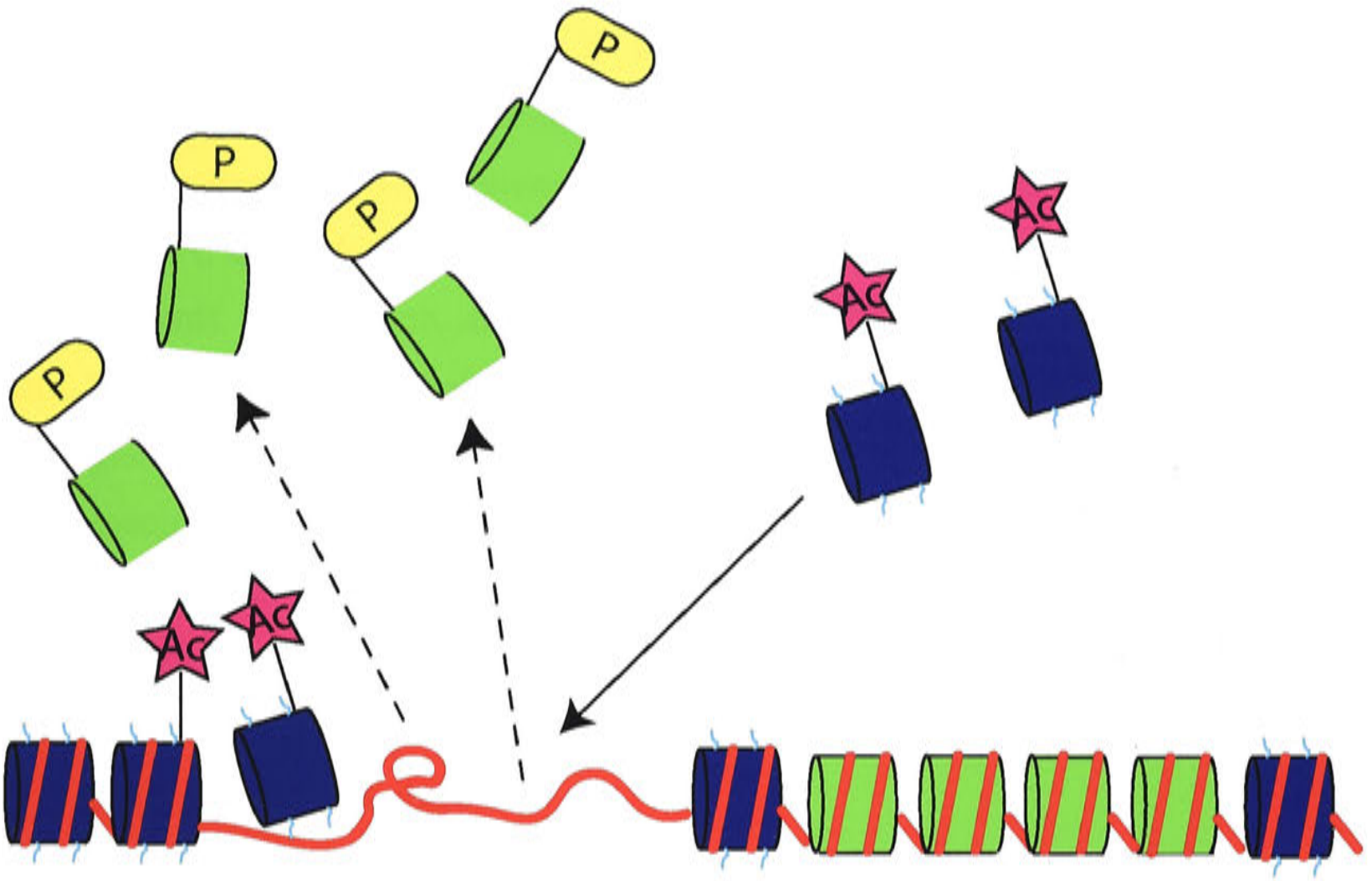

Figure 7.2: Chromatin remodeling during fertilization. Decondensation of the paternal pronuclei. Nucleosomes could be incorporated at specific sites in the sperm DNA. These nucleosomes would act as templates once decondensation occurs. The new nucleosomes could be positioned on the DNA in relation to the position of the pre-existing nucleosomes.

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APPENDICES

APPENDIX 1: SOLUTIONS

DOP-PCR

10µl of 10x Expand buffer (Roche, Germany)

10µl of 6MW primer (100ng/ul) (ID 66126 sequence = CCG ACT CGA GNN NNN NAT GTGG) (Telenius et al., 1992).

10µl 2mM dATP, dCTP, dGTP, and dTTP (Roche)

2µl of template DNA (flow sorted or microdissected chromosome)

1µl of 35U/µl Expand enzyme (Roche)

65µl of dH_20

DOP-PCR BIOTIN/DIGOXIGENIN

5µl of 10x Expand buffer

5µl of 6MW primer (100ng/ul)

5µl of solution containing 2mM of each of dGTP, dCTP, dATP

2µl of either biotin-16-dUTP or digoxigenin-11-dUTP

2µl of dTTP (2mM)

0.5ng of template DNA (produced from DOP-PCR reaction)

1µl of 35U/µl Expand enzyme

in a final volume of 50µl.

PCR program used was (94°C for 2 minutes) x1, (94°C for 2 minutes, 56°C for 1 min and 72°C for 3 minutes) x32, (72°C for 10 minutes) x1 and hold at 4°C.

PBS (pH 7.3-7.4)

$Na_2HPO_4.H_2O$	2.61g	AJAX chemicals
$NaH_2HPO_4.H_2O$	11.5g	AJAX chemicals
NaCl	8.7g	AnalaR
H_2O	1L	

20 x SSC

NaCl	175.3g (3M)	
$Na_3citrate.2H_2O$	88.2g (0.3M)	AJAX chemicals

H_2O	to 800ml	
1M HCl	to pH 7	AJAX chemicals
H_2O	to 1L	

1M Tris

Tris base	121.1g	AJAX chemicals
H_2O	1L	

Hybridizing Mixture

Deionized formamide	25ml	AJAX chemicals
20 x SSC pH 7	5ml	
Na_2HPO_4	2.3ml	
NaH_2PO_4	1.7ml	
50% Dextran Sulphate	10ml	
Denhart solution	0.5ml	

Denhart solution

Ficoll 450	1g	Pharmacia Biotech
BSA	1g	Sigma
H_2O	50ml	
Polyvonylpyrrolidone	1g	

Bromophenol blue

Bromophenol blue	0.025g	Sigma
Xylene cyanol	0.025g	International Biotechnologies
Ficoll 450	1.5g	
H_2O	10ml	

ϕX174 marker (No. IX) and λ marker (No.II)

Marker IX cleaved with HaeII	
or marker II cleaved with HindIII	100ul
Bromophenol blue	50ul
H_2O	350ul

10 x TBE

Tris base	108g	
Boric acid	55g	ICN Biomedicals
0.5M EDTA.H_2O	40ml	
H_2O	to 1L	

0.5M EDTA

$Na_2EDTA.H_2O$	186.1g	AJAX chemicals
H_2O	1L	

$MgCl_2$

$MgCl_2.6H_2O$	186.1g	AJAX chemicals
H_2O	1L	

3M NaOAc

Sodium citrate.$3H_2O$	20.3g	AJAX chemicals
H_2O	100ml	

4% paraformaldehyde

Parformaldehyde	40g	Sigma
PBS	400ml	
10M NaOH	1ml	
PBS	upto 1L	
(dissolve at 65°C)		

RNase (10mg/ml)

RNase	Roche
10mM Tris Cl	
15mM NaCl	
100°C for 15minutes	

Extraction buffer

Tris Cl (pH 8)	10mM
EDTA (pH 8)	0.1M
RNase	20ug/ml
SDS	0.5%

Blocking Solution (4 x SSC/0.2%Tween 20/1% BSA)

Tween	20µl
4 x SSC	10ml
BSA	0.1g

Runnning buffer

Tris	15.1g
Glycine	94g
SDS	50ml
H_2O	upto 1L

KCM buffer

KCl	9g
NaCl	1.16g
Tris HCl (pH 8)	10ml of 1M Tris HCl
EDTA	1ml of 500mM EDTA
Triton X-100	1ml

PRINS reaction mixture (10x reaction)

dATP, dCTP and dGTP	0.2mM @
dUTP labeled with Spectrum orange	0.2mM
KCl	50mM
Tris-Hcl pH 8.3	10mM
$MgCl_2$	1.5mM
BSA	0.01%
Taq DNA polymerase	1unit

Protease Inhibitor Cocktail

1. Pepstatin	5mg/ml in 100% DMSO	Sigma
2. Chymostatin	5mg/ml in 100% DMSO	Sigma
3. Antipain	5mg/ml in H_20	Sigma
4. Leupeptin	5mg/ml in H_20	Sigma
5. Aprotinin	10mg/ml in H_20	Sigma

STOCK: 50ul of 1-4 + 250ul of 5 + 9.55ml of H_20

Use 1:250 dilution

Store at -20°C in small aliquots

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Chromosomal painting detects non-random chromosome arrangement in dasyurid marsupial sperm

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Abstract

Chromosome arrangements have been studied in metaphase and interphase somatic cells and in sperm of many animal species, but there are conflicting data and it is still not clear whether chromosomes are arranged randomly or non-randomly. We used chromosome painting to reveal the positions of chromosomes in marsupial sperm. Marsupials are ideally suited for these studies because they have only a few large chromosomes. Here, we show that chromosomes occupy fixed positions in the immature and mature sperm of *Sminthopsis crassicaudata*. We suggest that the non-random arrangement of chromosomes in marsupial sperm may be important in establishing chromosome arrangement and patterns of gene activity within the developing embryo.

Introduction

Cytogeneticists have long speculated as to whether chromosomes are randomly positioned within cells or whether there is a fixed arrangement with some functional significance. Comings (1968) reviewed the considerable older evidence that chromosomes occupied fixed positions within interphase nuclei. However, the only direct evidence that chromosomes occupy specific positions within the nucleus was the inactive X chromosome in somatic cells of female eutherian mammals, which forms a genetically inactive (Lyon 1961) and cytologically heterochromatic sex chromatin body at the periphery of the nucleus (reviewed by Ohno 1967).

Early work used C-banding and the nucleolar organizing regions to identify chromosome positions within cells and show that, in some plants, the chromosomes are spatially organized within the haploid genome (reviewed by Bennett 1982).

In-situ hybridization and three-dimensional serial section studies later showed that chromosomes occupy specific domains within mammalian interphase cells (Lichter *et al.* 1988, Manuelidis & Borden 1988, Mosgoller *et al.* 1991, Leitch *et al.* 1994). Differences in position in different cell types (Manuelidis 1984) suggested a correlation between chromosome position and gene expression (reviewed by Heslop-Harrison & Bennett 1990, Haaf & Schmid 1991). Recently, chromosome painting was used to show that chromosomes have a conserved position in interphase cells and the mitotic rosette, with certain chromosome combinations always found together (Nagele *et al.* 1995, 1998, Koss 1998, Nagele *et al.* 1999).

Many studies of chromosome arrangements in animal sperm have been carried out over the last 40 years. The conclusion from early studies were almost evenly divided between random and non-random arrangement in sperm from insects, amphibians and birds. The wide variety of animal species studied, and the many different methods used perhaps accounted for the somewhat inconsistent results (Table 1).

However, there does seem to be some agreement from recent studies that chromosome arrangement in mammalian sperm is non-random. Watson *et al.* (1996) demonstrated by fluorescent and radioactive labeling of unique and repetitive sequences that chromosomes are arranged head to tail in the fibrillar sperm head of monotremes, and that particular chromosome regions occupy a consistent spatial position which was conserved between species. Meyer-Ficca *et al.* (1998) used chromosome painting to show that chromosomes have a non-random arrangement in

Table 1. Chromosome arrangement in animal sperm.

Species	Method	Chromosome positioning non-random/random	References
Insects			
Iceryine coccid	Feulgen staining	Non-random	Hughes-Schrader *et al.* 1946
Cave cricket	Polarized ultraviolet light	Non-random	Inoue & Sato 1962
Grasshoppers	Autoradiography	Random, tandem arrangement[a]	Taylor 1964
Plathelminthes			
Planarians	FISH: telomeric and rDNA probes	Non-random	Joffe *et al.* 1998
Amphibians			
Salamanders	Autoradiography	Random	Macgregor & Walker 1973
Anura	Q- and C-banding	Random	Schmid 1979
Birds			
Chicken	C-banding	Tandem arrangement	Dressler & Schmid 1976
	FISH: telomeric, repetitive and chromosome 6 probes	Random	Solovei *et al.* 1998
Mammals			
Monotremes: Echidna and Platypus	FISH: telomeric, radioactive *in-situ* for rDNA and gene specific probes	Non-random, tandem arrangement	Watson *et al.* 1996
Eutherians:			
Hamsters	Feulgen staining	Non-random, tandem arrangement	Douglas 1965
Rat	FISH: centromeric probes and chromosome painting	Non-random	Meyer-Ficca *et al.* 1998
Human	FISH: centromeric and telomeric probes	Centromeres, telomeres non-random	Zalensky *et al.* 1995, Luetjens *et al.* 1999,
	Chromosome painting	Non-random	Hazzouri *et al.* 2000

[a]Tandem arrangement: Chromosomes aligned one after another down the length of the sperm head.

rat sperm. Chromosome painting in human sperm showed that the X chromosome is localized preferentially in the anterior region and 18 in the posterior region (Luetjens *et al.* 1999, Hazzouri *et al.* 2000).

It has been suggested many times that the positions of chromosomes may play a role in gene expression and function, e.g. the Barr body. Aberrant positioning may be significant in some diseases (review by Qumsiyeh 1995). In epilepsy patients, the X chromosome was found to be repositioned in the neurons (Borden & Manuelidis 1988). The anterior position of the human X chromosome in sperm may explain why a higher rate of sex chromosome aneuploidies is seen in ICSI produced embryos (In't Veld *et al.* 1995). Decondensation in the anterior region of the sperm head, is inhibited by the acrosomal cap (Hewitson *et al.* 1996), and this may hinder the onset of the first mitosis in the zygote, leading to an elevated incidence of sex chromosome anomalies (Luetjens *et al.* 1999). Thus chromosome positioning in sperm may have a crucial role in the post-fertilization stages of development.

In our study we analyzed chromosome arrangements in marsupials. Marsupials constitute a second major group of extant mammals, having diverged from eutherians ('placental' mammals) around 130 million years ago (Clemens *et al.* 1989). They provide an ideal model to study chromosome arrangements, due to their large asymmetric sperm heads and their few large chromosomes. Although marsupial sperm DNA is packaged with protamines, they generally lack disulfide bonds between them (reviewed by Oliva & Dixon 1991, Retlief *et al.* 1995), making decondensation of sperm before *in-situ* hybridization unnecessary. DNA paints from microdissected or flow-sorted *Sminthopsis crassicaudata* chromosomes were hybridized to *S. crassicaudata* sperm to reveal the chromosome arrangement.

Materials and methods

Samples

Sminthopsis crassicaudata testes were supplied by Dr W. Breed, Department of Anatomy, Adelaide University, under permit number 10000572 (Victorian Department of Natural Resources and Development). Sperm were removed from each epididymis using a cut and squeeze method and fixed in 10× volume of methanol : acetic acid (3 : 1). The sperm suspension was then stored at −20°C until required for study. Upon thawing, a drop of the sperm suspension was placed onto glass slides and allowed to air dry at room temperature. The preparations were then treated with RNase (0.1 mg/ml, 90 min at 37°C) and pepsin (0.05%, 10 min, at 37°C) before procedures for FISH were carried out.

Fluorescence in-situ *hybridization (FISH)*

S. crassicaudata chromosomes were flow-sorted or microdissected (Rens *et al.* 1999, Toder *et al.* 2001). Chromosome-specific DNA was amplified and labeled with biotin or digoxigenin by degenerate oligonucleotide-primed PCR (DOP-PCR; Telenius *et al.* 1992). For fluorescence *in-situ* hybridization, 400 ng of labeled chromosome paints in 15 μl of hybridization mix (50% formamide, dextran sulfate 10%, in 2×SSC (saline–sodium citrate)) were used. Sonicated genomic *S. crassicaudata* DNA was used for suppression of repetitive sequences at a ratio of 1 : 50 (paint : suppressor DNA). The paint was denatured at 85°C for 6 min and preannealed for 20 min at 37°C. Sperm preparations were denatured at 70°C for 5 min. Hybridizations were carried out for 48 h at 37°C.

Post-hybridization washes consisted of six 2-min baths at 45°C, three in 50% formamide/SSC and three in 2×SSC. Immunodetection was carried out with antibiotin antibody raised in goat (3 : 500; Sigma) and rabbit antigoat antibody conjugated with fluorescein isothiocyanate (FITC; 1 : 100; Vector) or antidigoxigenin raised in mouse (3 : 500; Sigma) and antimouse conjugated with tetramethylrhodamine B isothiocyanate (TRITC; 1 : 100; Vector). Slides were mounted with 0.8 ng/μl DAPI (Sigma) in Vectashield (Vector).

Slides were viewed with a Zeiss Axioplan 2 microscope, and images captured by a liquid-cooled CCD camera. Images were produced with Photometrics SuperNu200 and NIH imaging software. Registration V1.1d2, Adobe Photoshop 3.0 and Adobe illustrator 7.0 were used to integrate the final images.

Results

Two distinct sperm morphologies were observed, corresponding to immature and mature sperm (Figure 1). Most were immature, presenting a T-shape where the sperm tail was inserted perpendicular to, and mid way along, the ventral surface of the sperm head (Figure 1a). About 10% of the sperm were mature, with the sperm head lying parallel to the sperm tail (Figure 1b). Mature sperm were removed from the epididymis where sperm maturation takes place in marsupials (Breed 1994). The attachment site of the tail to the sperm head consistently produced a strong signal due to non-specific antibody binding. The efficiency of hybridization to immature sperm (40–50%) was much greater than to mature sperm (20%), probably because of membrane changes or the chromatin condensation that occur during maturation.

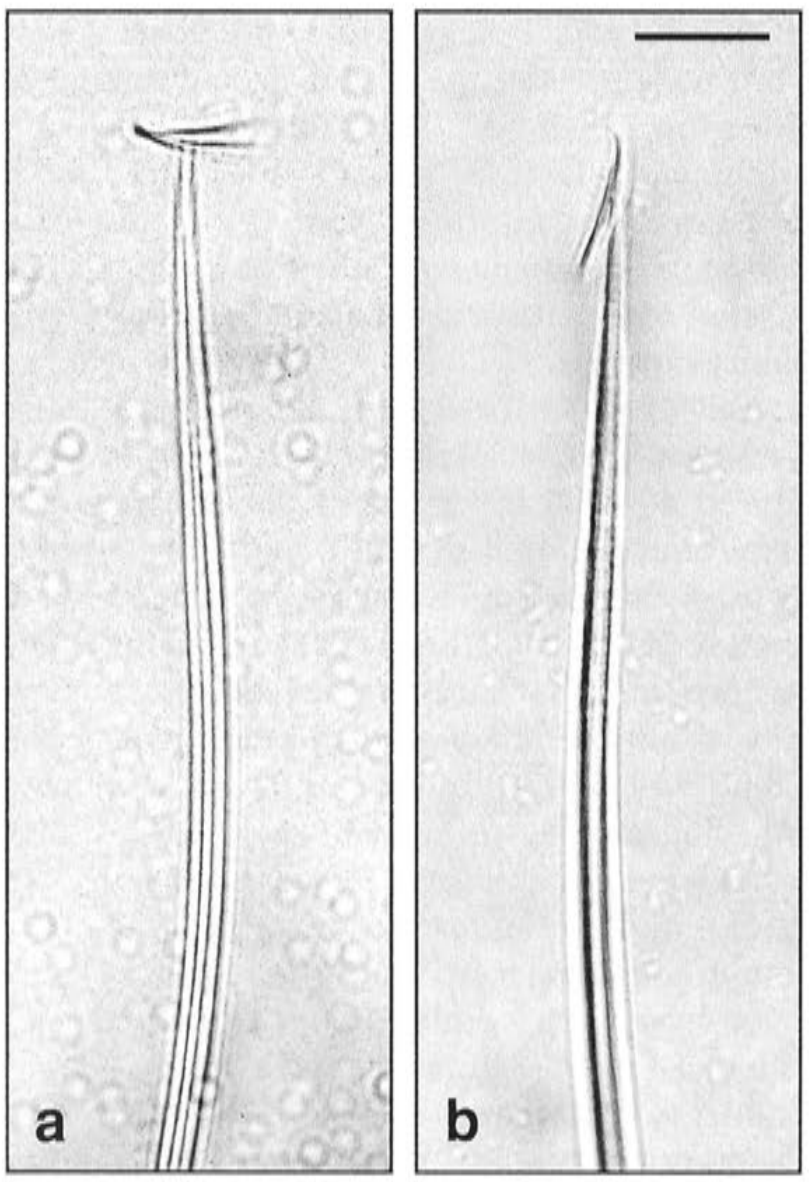

Figure 1. Immature corpus (**a**) and mature cauda (**b**) epididymal spermatozoa from *S. crassicaudata*. Note the T-shaped orientation of the nucleus (**a**) in relation to the tail which typifies immature *S. crassicaudata* sperm or those about to interact with the egg. Bar represents 10 μm.

Paints were available for each individual *S. crassicaudata* chromosome. They were each verified by hybridization to a single chromosome in mitotic cells. Each paint produced a single large and easily detectable signal in sperm.

The anterior/posterior and the dorsal/ventral asymmetry of the sperm head (Harding *et al.* 1982) were used as references for locating each hybridized chromosome paint within the sperm heads. Images of at least 30 sperm were captured for each paint to determine whether the chromosome position was consistent. For each paint, the signal produced was found non-randomly in a particular location with respect to the anterior and posterior ends of the sperm head, in both immature and mature sperm. Chromosome 3 was at the anterior region in 68% of cells, chromosomes 4 and 5 were found in the central region of the sperm in 70% and 78% of cells respectively, and chromosomes 1, 6 and 2 were found at the posterior region in 68%, 90% and 73% of cells, respectively (Figure 2; Table 2). Signals were frequently bipartite in sperm in which the tail was inserted along the upper face e.g. chromosome 4 in Figure 2. Variation in position of the observed signal may reflect actual chromosome position variation, or may be due to limitations of the two-dimensional equipment used and the variation in orientation of the sperm examined. For example, chromosome 3 was unambiguously at the anterior region in 68% of sperm, whereas, in the other 32%, they were distributed evenly between the central and posterior regions. Central localizations may, however, result from viewing the sperm from the top and non-specific binding may account for at least some of the signals that appeared to be in the posterior region. Misclassification of the anterior and posterior of the sperm could account for other mislocalizations.

The X and Y chromosomes occupied identical positions in X-bearing and Y-bearing sperm, both lying within the central region of the sperm head (Figure 2). The signal at the attachment site of the tail sometimes interfered with the recognition of the X or Y chromosome paints. It was therefore necessary to study sperm which had no tail or had

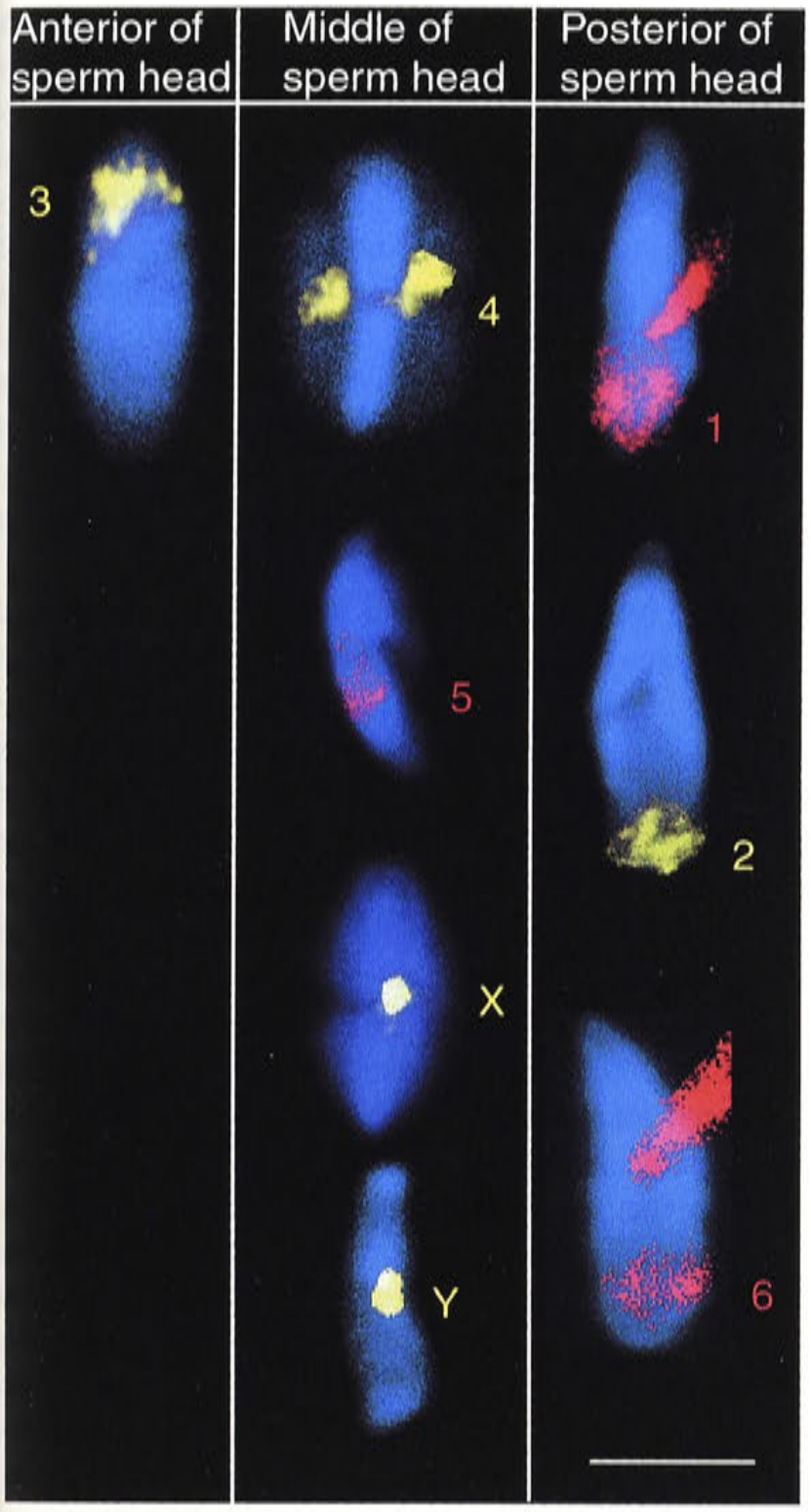

Figure 2. Chromosome positions in *S. crassicaudata* sperm revealed by hybridization with *S. crassicaudata* single chromosome paints. Chromosome 3 was usually found at the anterior end of the sperm nucleus, chromosomes 4, 5 and X/Y in the middle of the sperm nucleus, and chromosomes 1, 2 and 6 at the posterior end of the nucleus (red = digoxigenin, yellow = biotin). Bar represents 5 μm.

a tail inserted at different angles to distinguish the position of the X and Y chromosome.

Double labeling with two chromosomes was used to determine whether the relative positions of chromosomes were consistent and to allow a chromosome order to be deduced. Each combination gave consistent relative positions (Figure 3). For instance, chromosome 6 was always

Table 2. Non-random chromosome positioning.

Chromosome	Anterior	Medial	Posterior	Total
1	11 (17%)	10 (15%)	**45 (68%)**	66
2	3 (10%)	5 (17%)	**22 (73%)**	30
3	**34 (68%)**	8 (16%)	8 (16%)	50
4	12 (20%)	**41 (70%)**	6 (10%)	59
5	3 (6%)	**40 (78%)**	8 (16%)	51
6	1 (3%)	**27 (90%)**	2 (7%)	30
X/Y	4 (6%)	**57 (88%)**	4 (6%)	65

observed to be anterior to chromosome 2. Double labeling gave an unambiguous anterior to posterior chromosome order of 3, 4, 5, X/Y, 1, 6 and 2. Figure 4 summarizes our interpretation of the positions of chromosomes in *S. crassicaudata* sperm based on single and double labeling experiments. The bipartite signal in sperm viewed from the ventral surface suggests that chromosomes are arranged in a horseshoe shape around the insertion point of the tail.

Discussion

Chromosome painting allowed us to establish unequivocally that chromosomes occupy specific spatial domains in *S. crassicaudata* sperm. This result is consistent with the conclusion that

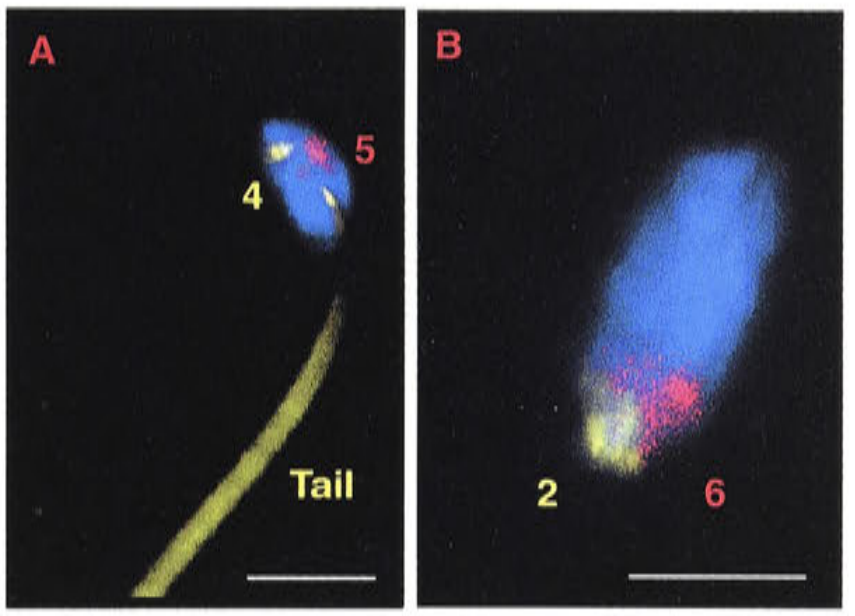

Figure 3. Two-color chromosome painting was used to determine chromosome positions relative to each other. Chromosome 4 was always anterior to chromosome 5 in the center of the sperm head (**a**); chromosome 6 was always anterior to chromosome 2 at the posterior of the sperm head (**b**). Bar represents 10 μm (**a**) and 5 μm (**b**).

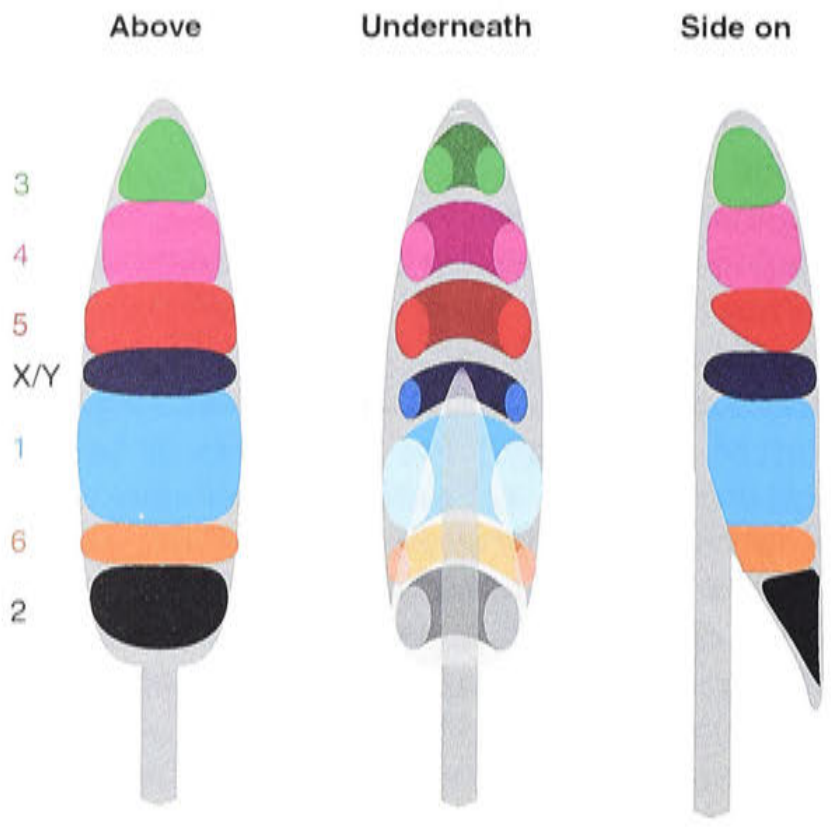

Figure 4. Model of chromosome positions in *S. crassicaudata* sperm.

chromosomal arrangement is non-random in planaria (Joffe *et al.* 1998), iceryine coccids (Hughes-Schrader *et al.* 1946), monotremes (Watson *et al.* 1996), rats (Meyer-Ficca *et al.* 1998), and humans (Luetjens *et al.* 1999), but not with the findings of an apparent random positioning in amphibians (Schmid 1979) and chickens (Solovei *et al.* 1998).

The inconsistent findings may be due either to *bona fide* differences between species or to differences in methodology. Early studies used Feulgen staining (Hughes-Schrader *et al.* 1946), autoradiography (Taylor 1964) and C-banding (Schmid 1979). These techniques detect classes of chromatin, allowing the general organization of chromosomes to be studied (i.e. tandem arrangements, or chromosomes bundled together) but not recognition of specific chromosomes within the sperm. More recent studies, including work by Zalensky *et al.* (1995), Watson *et al.* (1996) and Solovei *et al.* (1998), used hybridization of specific DNA probes to identify the positions of telomeric, centromeric, rDNA and specific genes in sperm. While these probes may give a clearer answer to the question of non-random chromosome organization, they do not provide an overall picture of chromosome domains within the sperm. For instance, Solovei *et al.* (1998) could detect the position of only chromosome 6 (detected by a unique sequence probe Gd/6) and the Z chromosome (detected by the CZMR repeat which is present also on chromosomes 1 to 4). This incompleteness and ambiguity weakens the conclusion that the chromosomes are arranged randomly. Ambiguity was, to a lesser extent, a problem with identifying two rDNA clusters by size in monotreme sperm, although several unique sequences ensured that at least four chromosomes could be identified (Watson *et al.* 1996).

Fixatives used within the experiments can affect nucleus organization (Skaer & Whytock 1976). For example methanol and acetic acid (3 : 1) used here causes dehydration and flattening of the cell nucleus on the slide. The overall effect on chromosome position is not likely to be significantly changed although it may influence the position of a gene within the chromosome domain (Kozubek *et al.* 2000).

One of the high resolution methods used to study nuclear organization within sperm is chromosome painting. The paints are chromosome specific and hybridize over the whole chromosome (reviewed by Ferguson-Smith 1997, Rens *et al.* 1999) so that each chromosome can be identified in its entirety within the sperm.

Our results, together with earlier studies on eutherian and monotreme mammals indicate that a non-random chromosome order within sperm is a conserved characteristic of all three extant mammal groups. It remains to be seen whether this is the case in lower vertebrates and invertebrates, although it would seem unlikely that such a fundamental property as chromosome arrangement in sperm would differ among animal classes. Since the available data on chromosome arrangement in insects and planarians cannot be readily evaluated because of the differences in techniques, chromosome painting of sperm in some of these species would be useful to determine whether apparent random chromosome arrangements in invertebrates are real or artefactual.

The position of the X chromosome is of particular interest because of the chromosome wide inactivation of the X. The X chromosome lies in the anterior region of the sperm head in humans (Luetjens *et al.* 1999) and monotremes (Watson *et al.* 1996). In *S. crassicaudata*, however, the X chromosome is positioned in the middle region

of the sperm nucleus directly above the point of insertion of the sperm tail in the ventral surface of the sperm head. In humans and monotremes, initial interaction between the sperm and the egg at fertilization is via the anterior edge of the sperm head (reviewed by Bedford 1991). In *S. crassicaudata*, however, the sperm head rotates back to its immature T-shape position immediately prior to sperm–egg interaction (reviewed by Rodger 1991, Breed 1994). Thus, in all three published studies (humans, (Luetjens *et al.* 1999) monotremes (Watson *et al.* 1996) and *S. crassicaudata* (marsupials; this study)), the position of the X chromosome corresponds to the position of the first contact between the sperm head and the egg at the time of fertilization (Figure 5). Thus, the X chromosome enters the egg early during fertilization in all three mammal groups. It is tempting to speculate that the conserved position of the X chromosome and its early entry into the egg at fertilization may have a role in X chromosome expression and inactivation in mammals. This may be particularly significant in marsupials, in which the inactive X is always paternal in origin and may therefore be delivered by sperm in an inactive state. If chromosome position in sperm is functionally significant, we would expect to see that it is conserved in related species. This prediction is currently being tested.

It will be interesting to study how this non-random chromosome arrangement within the sperm is set up at meiosis. Are chromosomes recognized in meiosis and moved into specific positions? Or does meiosis simply replicate a pre-existing order in spermatocytes? It will be especially interesting to know whether the non-random arrangement of chromosomes in *S. crassicaudata* sperm is important for setting up chromosome arrangement in the embryo and therefore significant for gene expression in the developing embryo.

Acknowledgements

We thank Doctor W. Breed, Department of Anatomy, Adelaide University, for supplying *S. crassicaudata* tissue and Iole Barbieri, Department of Genetics, LaTrobe University, for supplying *S. crassicuadata* metaphase preparations. Marta Svartman was supported by a fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), and the work was funded

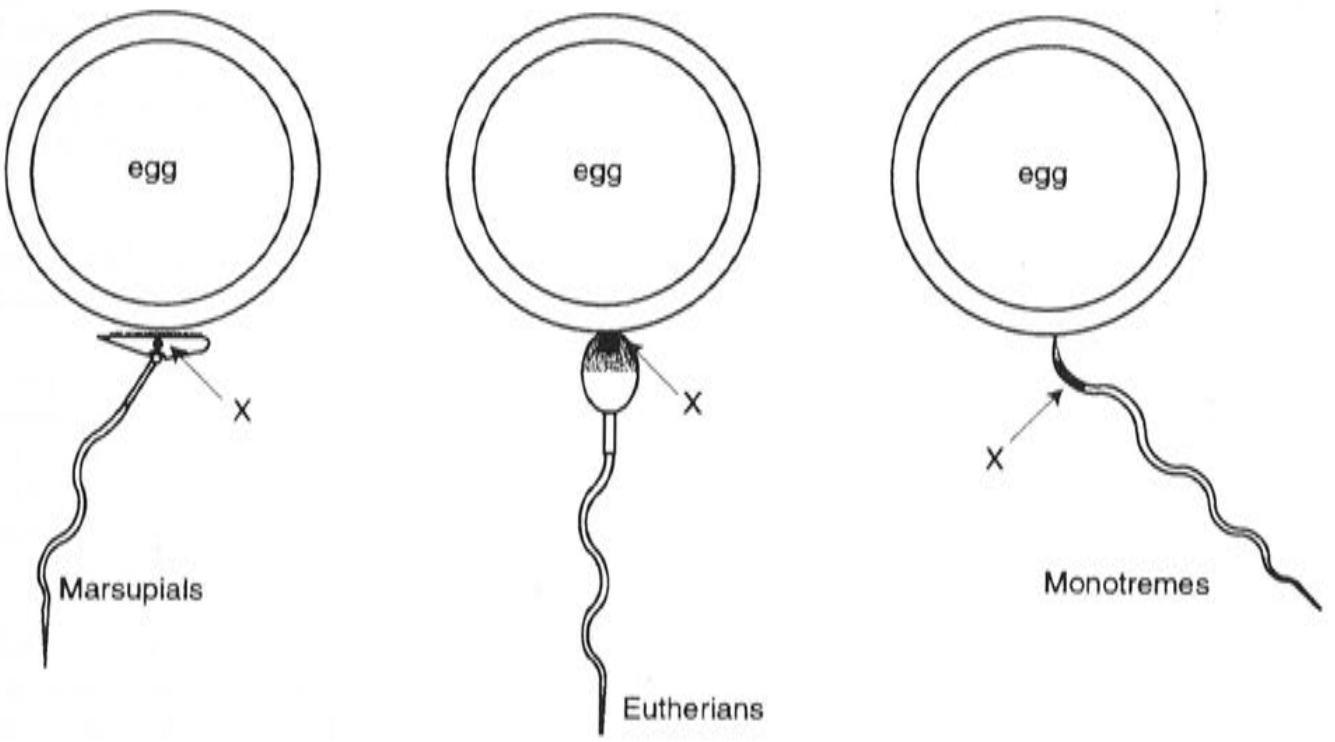

Figure 5. Conserved position of the X chromosome in relation to fertilization. In eutherians (humans) and monotremes (platypus or echidna), the X chromosome is positioned in the anterior of the sperm head (Luetjens *et al.* 1999, Watson *et al.* 1996). In *S. crassicaudata*, the X chromosome lies at the center of the sperm head which is also close to the sperm–egg association point which is central in many marsupial groups.

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Chromosome Research 9: 260, 2001.

Announcements

THE 5TH INTERNATIONAL SYMPOSIUM ON CHROMOSOMAL ABERRATIONS

– Perspectives for the 21st Century –

Awaji Yumebutai International Conference Center, Awaji Island, Hyogo
October 26–28, 2001

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Co-organized by: Mammalian Mutagenicity Study Group/Japanese Environmental Mutagen Society (JEMS·MMS); The Society of Chromosome Research; The Japan Radiation Research Society

Calling for papers: 30 posters will be accepted

Correspondence: Makoto Hayashi, NIHS, Tokyo, Japan, hayashi@nihs.go.jp/Günter Obe, University of Essen, Essen, Germany, guenter.obe@uni-essen.de

Provisional programme

October 26

16:00 Registration

17:30–20:00 5th ISCA/JEMS·MMS 20th anniversary meeting

17:30–17:45 Opening remarks by *Dr. M. Ishidate, Jr.*

Chairperson: Dr. Ishidate

17:45-18:45 Keynote lecture-1
Dr. A.T. Natarajan
Chromosomal aberrations: Past, present and future

19:00–20:00 Keynote lecture-2
Dr. J.M. Parry
Biological relevance of aneuploidy

20:00– Free night

October 27

8:30-up to the end of the symposium Exhibition of 30 posters

8:30–12:30 Session-1 Chairpersons: Drs. AT Natarajan & M Hayashi

1-1 ***Dr. Hideyuki Tanabe***
Analyses of lymphoblastoid cell nuclei by using a multicolor 3D-FISH technique: chromosome topology and evolutionary considerations

1-2 ***Dr. Hisaji Maki***
Spontaneous loss of heterozygosity in diploid *S. cerevisiae* cells: Chromosome aberrations induced in *rad50, rad51*, and *rad52* mutants

10:30-11:00 Coffee break

1-5 ***Dr. Yutaka Ishii***
DSB repair in G1 and G2 in chromosomal aberration formation

1-6 ***Dr. Predrag Slijepcevic***
Telomeres and mechanisms of the formation of chromosomal aberrations

1-3 ***Dr. Carol Griffin***
Aneuploidy and centrosome activity in homologous recombination repair mutants *irs1* and *irs1SF*

1-4 ***Dr. Günter Obe***
DSB repair and chromosomal aberrations

1-7 ***Dr. Jordi Surralles***
Chromosomal aberrations, telomeres, and end-fusions: insights from Fanconi anemia

12:30–14:00 Lunch

14:00–16:30 Session-2 Chairpersons: Drs. G Obe & T Sofuni

2-1 ***Dr. Jan Boei***
Complex chromosomal aberrations

2-2 ***Dr. Takatomo Sato***
Application of M-FISH for analysis of chromosomal aberrations

2-3 ***Dr. Jim Tucker***
Persistence of translocations

2-4 ***Dr. Jia Cao***
Study on the changes of chromosomal aberrations and micronuclei as biological indicator for nasopharynx cancer patients receiving radiation therapy

2-5 ***Dr. Masao S. Sasaki***
Generalized chromosome-based biodosimetry system

17:00–19:00 Banquet

20:00–22:00 Poster session

October 28

8:30–12:00 Session-3 Chairpersons: Drs. J.M. Parry & Y. Ishii

3-1 (8:30–9:00) ***Dr. Micheline Kirsch-Volders***
Importance of detecting aneuploidy and polyploidy versus chromosome aberrations

3-2 (9:00-9:30) ***Dr. Yujiroh Kamiguchi***
Radiation- and chemical-induced structural chromosomal aberrations in human spermatozoa

10:00–10:30 Coffee break

3-4 (10:30-11:00) ***Dr. Shin-ichi Sonta***
Transmission of structurally abnormal chromosomes: Meiotic segregation and the fate of unbalanced gametes in mammals

3-5 (11:00-11:30) ***Dr. William Morgan***
Delayed chromosomal instability: The role of DNA damage, bystander effects, and recombination-mediated processes

3-3 (9:30–10:00) ***Dr. Ilse-Dore Adler***
Induction of aneuploidy in mammalian male germ cell using the sperm-FISH assay

3-6 (11:30–12:00) ***Dr. Michael Fenech***
New developments in the cytokinesis-block micronucleus assay and the HUMN project

12:00 Closing remarks by *Dr. Takaji Ikushima* and adjourn

APPENDIX 3:

Greaves, I. K., Greaves, I. K., Rens, W., Ferguson-Smith, M. A., Griffin, D. Graves, J. A. M. (2003). Conservation of chromosome arrangement and position of the X in mammalian sperm suggests functional significance. *Chromosome Res,* **in press.**

Conservation of chromosome arrangement and position of the X in mammalian sperm suggests functional significance

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Running head: Conserved chromosome arrangement in mammal sperm

SUMMARY

We used chromosome painting to show directly that chromosomes occupy fixed positions in the nuclei of mammal but not chicken sperm. We found that the positions of homologous chromosomes are conserved in sperm of two marsupial species that diverged 50–60 million years ago. We also discovered that the X chromosome lies in the region that makes first contact with the egg in marsupial and monotreme mammals, as well as eutherians, and suggest that this position may be related to its propensity for inactivation, and its high rate of loss from ICSI embryos. We propose that nuclear architecture in sperm is important for spatial chromatin differentiation and normal development of the fertilized egg, and evolved along with mammal-specific regulatory systems such as X inactivation and genomic imprinting.

INTRODUCTION

The level of nuclear organization within interphase and metaphase cells has been studied over decades. Early investigators suggested that chromosomes occupy defined positions within the cell nucleus, but little direct evidence was available to support such claims. Apart from centromeric and heterochromatic associations, the peripheral position of the sex chromatin body (representing the inactive X chromosome at interphase) in cells of eutherian females was the most convincing evidence for a non-random position of a chromosome (Barr *et al.*, 1949; Comings, 1968).

More recently, the use of *in situ* hybridization to identify individual chromosomes or regions at interphase, has led to major advances in the analysis of nuclear organization. Chromosome painting and confocal microscopy of mammalian interphase cells showed that chromosomes occupy discrete territories within the nucleus that are non-randomly arranged (Lichter *et al.*, 1988; Manuelidis *et al.*, 1988; Nagele *et al.*, 1999) in a radial configuration (Cremer *et al.*, 2001a; Habermann *et al.*, 2001). Chromosome position varies through the cell cycle (Bridger *et al.*, 2000), and is influenced by its gene density and transcriptional activity (Boyle *et al.*, 2001). Gene-rich chromosomes such as human chromosome 19 lie towards the interior of the cell nucleus, whereas gene-poor chromosomes (such as human chromosome 18) and the inactive X chromosome lie towards the periphery of the cell nucleus (Croft *et al.*, 1999). Cross-species chromosome painting showed that this arrangement of chromosome 18 and 19 homologues is shared by other primate species

(Tanabe *et al.*, 2002). This arrangement accords with the position of early replicating (active) DNA towards the center and late replicating (inactive) at the periphery of the nucleus (Nogami *et al.*, 2000).

Higher order organization is also seen within the chromosome territory, with active genes located towards the periphery and inactive genes towards the interior. (Cremer *et al.*, 2001b; Francastel *et al.*, 2000). In mouse lymphocytes gene silencing is associated with the repositioning of genes close to centromeres (Brown *et al.*, 1997). Thus it is now clear that there is a high level of nuclear organization within the cell nucleus at interphase, and some clues that this arrangement is conserved and functionally important.

To know how this conserved arrangement is transmitted between generations, it would be useful to study the arrangement of chromosome in gametes. Chromosome organization has been studied in sperm since the 1940s (Hughes-Schrader, 1946; Taylor, 1964; Douglas, 1965), but data are conflicting, with evidence of non-random or random positions in different animals, using different techniques.

There is evidence for non-random positions of chromosomes in all three extant mammalian groups. In monotremes (the egg-laying platypus and echidna) *in situ* hybridization using fluorescent telomere probes and radioactive gene probes demonstrated a tandem arrangement of chromosomes in the fibrillar spermhead, with the X at the anterior. Chromosome painting in a dasyurid demonstrated a strikingly non-random arrangement of chromosomes (Greaves *et al.*, 2001). Marsupial material is ideal because of the asymmetric and relatively uncondensed spermheads, and the low chromosome number. Painting patterns in the sperm of eutherian ("placental") mammals have been harder to interpret because the spherical spermhead offers few landmarks. Also methods to disrupt disulfide bonds in the protamines of the highly condensed eutherian sperm nucleus to allow *in situ* hybridization may alter nuclear organization of the sperm nucleus (Oliva *et al.*, 1991). However, there are reports of non-random organization of chromosomes in eutherian sperm (Luetjens *et al.*, 1999; Meyer-Ficca *et al.*, 1998). For instance, chromosomes 2 and 12 were shown to have non-random positions in rat sperm (Meyer-Ficca *et al.*, 1998). Of particular interest is the finding that in human sperm the X chromosome is preferentially located in the anterior (Hazzouri *et al.*, 2000; Luetjens *et al.*, 1999).

The opposite result has been obtained in birds. From the position of telomeric and repetitive probes in the fibrillar chicken sperm, it was concluded that chromosomes had a random arrangement in sperm (Solovei *et al.,* 1998).

It seems remarkable that a characteristic as fundamental as chromosome organization in sperm should be so different in mammalian and bird sperm, particularly as sperm morphology and development is very similar between birds and monotremes. We therefore used chromosome-specific paints to marsupial, monotreme and chicken sperm to directly observe whether non-random arrangement is a mammal specific trait. We also compared chromosome arrangement in two distantly related marsupials with almost identical karyotypes to determine to what extent the arrangement is conserved and to assess its significance.

Monotreme and marsupial mammals diverged from eutherian mammals 170 and 130 million years ago respectively (Kirsch *et al.,* 1997). We studied an Australian marsupial, the Southern hairy nosed wombat (*Lasiorhinus latifrons)*, distantly related to the previously studied fat tailed dunnart (*Sminthopsis crassicaudata*). These two species diverged at least 50 Million years ago (Kirsch *et al.,* 1997), but both retain an almost identical karyotype closely related to the ancestral 2n=14 basic marsupial karyotype (Rofe and Hayman, 1986). We also studied chromosome position in the sperm of a monotreme (the platypus, *Ornithorynchus anatinus*), and the chicken (*Gallus domesticus*) both of which have fibrillar spermheads with a clearly defined anterior/posterior axis.

MATERIALS AND METHODS

Samples and cell culture

Wombat sperm was collected through electro-ejaculation under permit #C23313-01, S.A. National Parks and Wildlife Research and permit #M46-93, University of Adelaide Animal Ethics, Department of Anatomical Science, by Dr. D. Taggart (Adelaide University). Platypus testis was obtained from Environment ACT (Canberra) under Environment ACT permit #LI 2001204. Chicken testis was obtained under Environment ACT permit #LI 2001204 and ANU Animal Ethics R.CG.02.00. Isolated sperm were fixed in 3:1 methanol-glacial acetic acid and air dried. Sperm preparations were then either used immediately for the experiment or stored at -20°C. Since marsupial sperm lack disulfide bonds between protamine molecules chromosome paints can be hybridized without any decondensation

treatment.

Fibroblast cultures of wombat, platypus and chicken were established by Mrs Pat Miethke from skin, toe-web and embryo respectively. Cells were grown in 10% DME supplemented with 10% fetal calf serum and in the presence of Streptomycin and Penicillin. The cells were harvested with 50 – 75ng/ml Colcemid for 2 - 4 hours, centrifuged, fixed in 3:1 methanol-glacial acetic acid, dropped onto acid washed slides and air dried.

Paint preparation and probes

Chromosomes were flow-sorted from diploid fibroblasts of wombat, platypus and chicken as described previously (Rens, *et al.,* 1999). A Telomere-specific $(CCCTAA)_7$ oligonucleotide end labeled with Cyanine 3 (Cy3) was obtained from Geneworks (Adelaide, Australia).

Chromosome-specific DNA was amplified and labeled with biotin or digoxigenin by degenerate oligonucleotide-primed PCR (DOP-PCR). 400ng of labeled chromosome paints in 15µl of hybridization mix (50% formamide, dextran sulfate 10%, in 2xSSC) was used for fluorescence *in situ* hybridization (FISH). Sonicated genomic DNA in 50 fold excess was used for suppression. Chromosome-specific paints were denatured at 85°C for 6 minutes and preannealed for 20 minutes at 37°C.

In situ hybridization

Sperm preparations were denatured at 70°C for 5 minutes. Hybridizations were carried out for 48 hours at 37°C. Post hybridization washes consisted of six 2 minute baths at 45°C, three in 50% formamide/SSC and three in 2xSSC. Immunodetection was carried out with anti-biotin antibody raised in goat (3:500; Sigma) and rabbit anti-goat antibody conjugated with fluorescein isothiocyanate (FITC; 1:100; Vector) or anti-digoxigenin raised in mouse (3:500; Sigma) and goat anti-mouse conjugated with tetramethylrhodamine B isothiocyanate (TRITC; 1:100; Vector). Slides were mounted with 0.8ng/µl DAPI (Sigma) in Vectashield (Vector).

Slides were viewed with a Zeiss Axioplan 2 microscope, and images captured by a Photometrics liquid cooled CCD camera. Images were produced with SuperNu200 and NIH imaging software. Registration V1.1d2, Adobe Photoshop 3.0 and Adobe Illustrator 7.0 were used to integrate the final images.

RESULTS

Chromosome arrangement in wombat sperm

Chromosome-specific paints derived from flow-sorted wombat chromosomes were checked by hybridizing to metaphase spreads of a line of female wombat cells (not shown). All paints hybridized to a single wombat chromosome except for the small chromosome 6 (which provided an unacceptably weak signal). Paints were then hybridized onto wombat sperm, and for each chromosome the position of signal was recorded in at least 15 sperm. The asymmetrical (hook shaped) sperm head of the wombat made it easy to determine positions of signals relative to anterior/posterior and dorso/ventral landmarks.

Painting with individual chromosome probes, and two differentially labeled probes in combination, showed that the positions of chromosomes were non-random (Fig 1). For each paint, signals were found in consistent positions in 75-95% of sperm (Fig. 2), variation probably reflecting two-dimensional observations on sperm fixed at different angles. The order of chromosomes from the anterior to the posterior of the wombat sperm head was 3 - 4 - 5 - X - 1 - 2.

Conservation of chromosome arrangement in distantly related marsupials

The wombat belongs to a different marsupial order than the previously studied dunnart (Greaves *et al.*, 2001), but its karyotype is virtually identical. The hook-shaped wombat sperm morphology is somewhat different from the arrow-shaped dunnart sperm, but both have clearly defined anterior-posterior and dorso-ventral axes.

We therefore assembled the data from wombat and dunnart sperm (Greaves *et al.*, 2001) in the same manner so that we could directly compare the positions of homologous chromosomes (Figs 1, 2). The positions of all chromosomes were absolutely conserved. For example, chromosomes 4 and 5 were both located in the medial region of the sperm head in both species, with chromosome 4 slightly more anterior (Fig. 1a). Chromosome 2 lay in the posterior region of the sperm head in both species (Fig. 1b). The chromosome order in both species was 3 - 4 - 5 - X - 1 - (6) - 2.

Telomeric probe hybridized to the extremities of wombat and dunnart chromosomes at metaphase. Discrete signals were peripherally localized in dunnart sperm. No signal

could be observed in wombat sperm, but a peripheral location of discrete signals was observed for sperm of the closely related koala.

The same chromosome arrangement is therefore present in sperm of these distantly related marsupial species (Fig. 1c).

Chromosome arrangement in monotreme sperm

Two flow-sorted platypus chromosomes were used as probes to define chromosome position in platypus sperm. Paints prepared from the X chromosome and a small autosome, one of pairs 17-21, that are indistinguishable by G-banding (Wrigley *et al.,* 1988), were checked by painting onto mitotic metaphase spreads. The X paint produced signal on the X, as well as on the unpaired element (the putative Y) that pairs with the X at meiosis, and the autosomal paint produced signal on a small pair of autosomes (Rens et al, unpublished).

These paints were applied to platypus sperm. The fibrillar sperm nucleus and the pointed apex of the sperm made it easy to determine the position of each signal. The positions of each chromosome were scored in at least 30 sperm. Tight localization and fixed position was strikingly obvious for each signal. The X chromosome was found to have an apical position in 75% of platypus sperm (Fig. 2). This corroborates the finding of Watson et al. (1996), who used radioactive *in situ* hybridization to show that genes mapped to the X chromosome were preferentially located at the anterior of the sperm head. The platypus autosomal paint showed preferential localization of a tight signal at the very posterior end of the sperm head (Fig 3) in more than 80% of sperm (Fig. 3).

Telomeric probes produced signal at each end of each chromosome at metaphase. In sperm, signal was distributed as 25-30 dots or bands along the length of the spermhead (not shown) as previously described (Watson *et al*, 1996). This suggests that the 26 chromosomes of haploid sperm are arranged in tight territories along the sperm, overlapping only at their telomeres.

Chromosome arrangement in chicken sperm

The chicken karyotype is composed of nine macrochromosomes and many tiny microchromosomes. Four chicken flow-sorted macrochromosomes (2, 8, 9 and Z) were painted to chicken sperm, and the position of each scored in at least 30 sperm. Position relative to the ends and middle of the fibrillar spermhead was easy to score, but the anterior and posterior ends of the spermhead could not be reliably distinguished in FISH

preparations. Thus position relative to the middle was recorded for each chromosome (Fig 2).

Chromosome localization in the fibrillar spermhead was strikingly different from that in monotreme sperm. The signal was much more diffuse, sometimes stretching over a quarter of the spermhead. None of the chromosome signals were fixed within the sperm head (Fig 2). Although all showed a slight preference for the ends of the sperm heads, more than half of the signals were distributed over other locations. Multicolour painting of two chromosomes clearly showed that their relative positions were inconsistent in different sperm (Fig 3). This was the case for all of the chicken chromosomes, including the sex chromosome (Z), and chromosome 9.

Telomeric probe hybridized to chicken fibroblasts unexpectedly proved to label microchromosomes heavily, so that telomere signal was swamped. This probe was therefore used to detect the position of microchromosomes in chicken sperm. Again, the position of signal varied between sperm, but was frequently clumped in the medial region (Fig 3). We conclude that microchromosomes tend to lie in the centre of the sperm and macrochromosomes toward the extremities.

Our finding of inconsistent chromosome position in chicken sperm directly demonstrates that a conserved non-random arrangement of chromosomes in sperm is a feature of mammals, but not birds.

DISCUSSION

There are three important conclusions from our work. Firstly, a non-random chromosome arrangement in sperm seems to be a feature of all groups of mammals, but not of birds. Secondly, the chromosome arrangement in marsupial mammals is highly conserved, suggesting that it is functionally significant. Thirdly, similarities in the location of the X chromosome in all mammals suggests functional significance.

Non-random chromosome arrangement is specific to mammal sperm

Our results directly demonstrate that chromosomes are tightly localized and positioned non-randomly in marsupial and monotreme sperm. Although chromosome positioning is less clear in eutherian sperm, there are indications that chromosome territories are constrained, and their arrangement in human and rat sperm is non-random (Meyer-Ficca et al. 1998; Luetjens et al. 1999). Thus a non-random arrangement of chromosomes seems to be a feature of mammalian sperm.

Remarkably, such a consistent chromosome order is quite lacking for chicken sperm. Our direct chromosome painting confirms this conclusion of Solovei et al (1998) from localization of a Z chromosome specific heterochromatin and a chromosome 6 probe. Although there is a tendency for macrochromosomes to occupy the end(s) of sperm and microchromosomes the middle, individual chromosomes occupied extended territories at any position within a sperm head. Combinations of two chromosomes showed inconsistent relative positions in sperm, from separate localizations at either end, to overlapping in the middle. The contrast between chromosome position in chicken and monotreme sperm is particularly telling, since sperm morphology and development is so similar in these animals (Lin et al. 2000). Whereas chromosomes occupied constrained territories at specific positions in platypus sperm, chromosomes are extended and unordered in chicken sperm.

Assuming that the unordered arrangement in chicken sperm is typical of other birds and reptiles, which have very similar karyotypes, an ordered arrangement of chromosome appear to be a mammal specific feature. Its evolution in mammals will need to be explained.

Chromosome arrangement in mammalian sperm is highly conserved

The two Australian marsupials *Sminthopsis crassicaudata* and *Lasiorhinus latifrons* (the dunnart and wombat) are ideal for testing the hypothesis that the arrangement of

chromosomes in sperm is highly conserved in evolution. Although these species are members of marsupial orders that diverged 50-60 million yeas ago (Kirsch et al, 1996), they both retain the 2n=14 karyotype that is considered ancestral in marsupials, and comparative painting shows that chromosomes differ by only four paracentric inversions (De Leo *et al.*, 1999).

The arrangement of homologous chromosomes in sperm nuclei was found to be identical in the two species. This implies that the arrangement of chromosomes has remained unchanged for at least 50 million years of marsupial evolution.

Position of sex chromosomes in sperm

The positions of the X and Y chromosomes relative to the acrosome are similar in sperm of all mammal groups. In monotremes, we observed that the X chromosome paint was localized at the apex of the platypus sperm head, as was previously implied by the radioactive *in situ* location of X-borne genes (Watson et al, 1996). Since this paint also detects the pairing partner of the X (the putative Y), this localization reflects both. Thus the sex chromosomes are located in the region nearest to the acrosome, and are presumably the first chromosomes to enter the egg on fertilization. In human sperm, too, the X chromosome has an anterior position in the nucleus (Hazzouri *et al.*, 2000). In the dunnart and wombat sperm, the X and Y chromosomes occupy a medial rather than anterior position. However, after a morphological transition to a T-shape during sperm maturation, this position, too, becomes the point of first contact with the egg on fertilization (Breed, 1994). In contrast, the chicken Z chromosome did not preferentially localize to the end of the fibrillar sperm, nor did chromosome 9.

The consistency of the X and Y chromosome position in sperm in monotremes, marsupials and eutherians implies that it has been a feature of mammals since the divergence of the three major mammal groups 170 million years ago. This conservation suggests that sex chromosome position in sperm serves an important function. One possibility is that X chromosome position is critical for establishing X chromosome inactivation. Paternal X chromosome inactivation (thought to be the ancestral mechanism) could result from compartmentalization and differential association with proteins that affect the subsequent activity of the X in the embryo.

Functional significance of the conserved chromosome arrangement

The conservation of chromosome arrangement in sperm suggests an important function in gametes. This function could be mechanical, having to do with the packaging of chromatin into sperm, or could be crucial is setting up the correct arrangement of chromosomes in the zygote. Chromatin undergoes many changes as it is packaged for spermiogenesis. During meiosis, there is a highly ordered substitution of histones for protamines, as well as sequence specific packaging of histones in sperm (Gatewood *et al.*, 1987; Oliva *et al.*, 1991). The ordered arrangement of chromosomes in mammalian sperm may produce, or be a product of, these processes.

Alternatively, the highly organized and conserved chromosome arrangement in sperm that we observed may be critical for transmitting imprinted inactivation states from parental gametes and establishing chromatin domains in the embryo. There are still few studies of chromosome behavior during and after fertilization and pronuclear fusion, but there is some indication that chromatin differences originating in the gametes persist in the embryo for several cell generations after fertilization. Differential methylation patterns and replication timing between paternal and maternal genomes can be detected up to the four cell stage (Haaf, 2001). Association of the embryonic (but not the adult) globin genes with histones rather than protamines in human sperm suggests that some chromatin domains arrive in the egg already primed for activity (Gardiner-Garden *et al.*, 1998). These epigenetic differences must be acquired during gametogenesis, perhaps as the result of differential chromatin packaging and positioning in sperm.

If the chromosome arrangement is so important in mammals, its absence in birds becomes even more mysterious. What forces could have selected for a tightly organized genome in sperm? One possibility is that X chromosome position is critical in establishing X chromosome inactivation, a mammal-specific control mechanism. Paternal X chromosome inactivation (thought to be the ancestral mechanism) could result from compartmentalization and differential association with proteins that affect the subsequent activity of the X in the embryo. The random localization of the Z chromosome is not surprising, given that, even if there is some form of dosage compensation, there seems to be no equivalent of X inactivation in birds (Ellegren, 2002).

The transmission of imprinted states of autosomal genes (again, a mammal-specific phenomenon), might also depend on the location of the region in sperm.

We suggest, therefore, that a tight and ordered localization of chromosomes in sperm evolved as a mechanism for transmitting and setting up paternal X inactivation and

genomic imprinting.

Chromosome arrangement and disease

The fixed positions of chromosome could be responsible for differential rates of somatic and germline chromosome abnormalities. For instance, a recurrent primary abnormality in chronic myleiod leukemia involves a translocation that brings together the *ABL* gene on chromosome 9 and *BCR* on chromosome 22. The significantly high frequency of this translocation can be explained by the location of both chromosome 9 and 22 towards the interior of the cell nucleus (Kozubek *et al.*, 1999).

The acrosomal juxtaposition of the X and Y chromosomes might also render them especially vulnerable to loss, explaining the high frequency of sex chromosome aneuploidy in mammals. In particular, the high frequency of sex chromosome loss from human embryos after intracytoplasmic sperm injection (ICSI) (In't Veld *et al.*, 1995) could be due to the delay of DNA replication at the apical region that contains the sex chromosomes, caused by the retention of the acrosomal cap (Hewitson *et al.*, 2000; Terada *et al.*, 2000).

If the arrangement of chromosomes is functionally significant, incorrect position might be expected to be associated with an abnormal phenotype. It is possible that some of the atypical sperm we saw were not just the result of unusual orientation but reflect a minority of sperm with an abnormal chromosome arrangement that may cause aberrations in fertilization or development. The abnormal position of the sex chromatin body in epileptic foci of epilepsy patients (Borden *et al.*, 1988) may indicate that internal relocation of the X chromosome (containing many genes involved in brain development and function; Zechner *et al.*, 2001) causes transcription of brain-specific genes from the inactive X chromosome.

Definition of the normal arrangement of chromosomes in human sperm may help to identify and correlate such abnormalities.

FIGURE LEGENDS

Figure 1. Position of chromosomes in marsupial sperm. (a) Homology of position is seen by double and single chromosome painting with probes to chromosome 4 (green), 5 (pink) and 2 (yellow) in dunnart and wombat sperm. (b) Comparison of the positions of homologous chromosomes in the arrow-shaped dunnart and the hook-shaped wombat sperm nuclei. Wombat chromosome 6 could not be satisfactorily detected.

Figure 2. Frequency with which chromosome-specific paints are anterior (blue), medial (green) or posterior (red) in sperm from the dunnart (a), the wombat (b) and the platypus (c). Because it was impossible to distinguish the two ends of chicken sperm, the position of chromosomes was recorded for three approximately equal parts; the two end regions (yellow), the two subterminal regions (pink), and two medial region (light blue).

Figure 3. Chromosome arrangement in the fibrillar sperm of platypus (left) and chicken (right). (a) Non-random position of the platypus X (above) and a group 17-21 autosome (below) in platypus sperm. (b) Random positions of the chicken chromosomes demonstrated by multicolour painting. Top: chromosome 8 (pink) and chromosome 9 (green); bottom: the Z chromosome (pink) and chromosome 9 (green) in chicken sperm. (c) Position of microchromosomes, labeled with telomere-specific probe, in chicken sperm.

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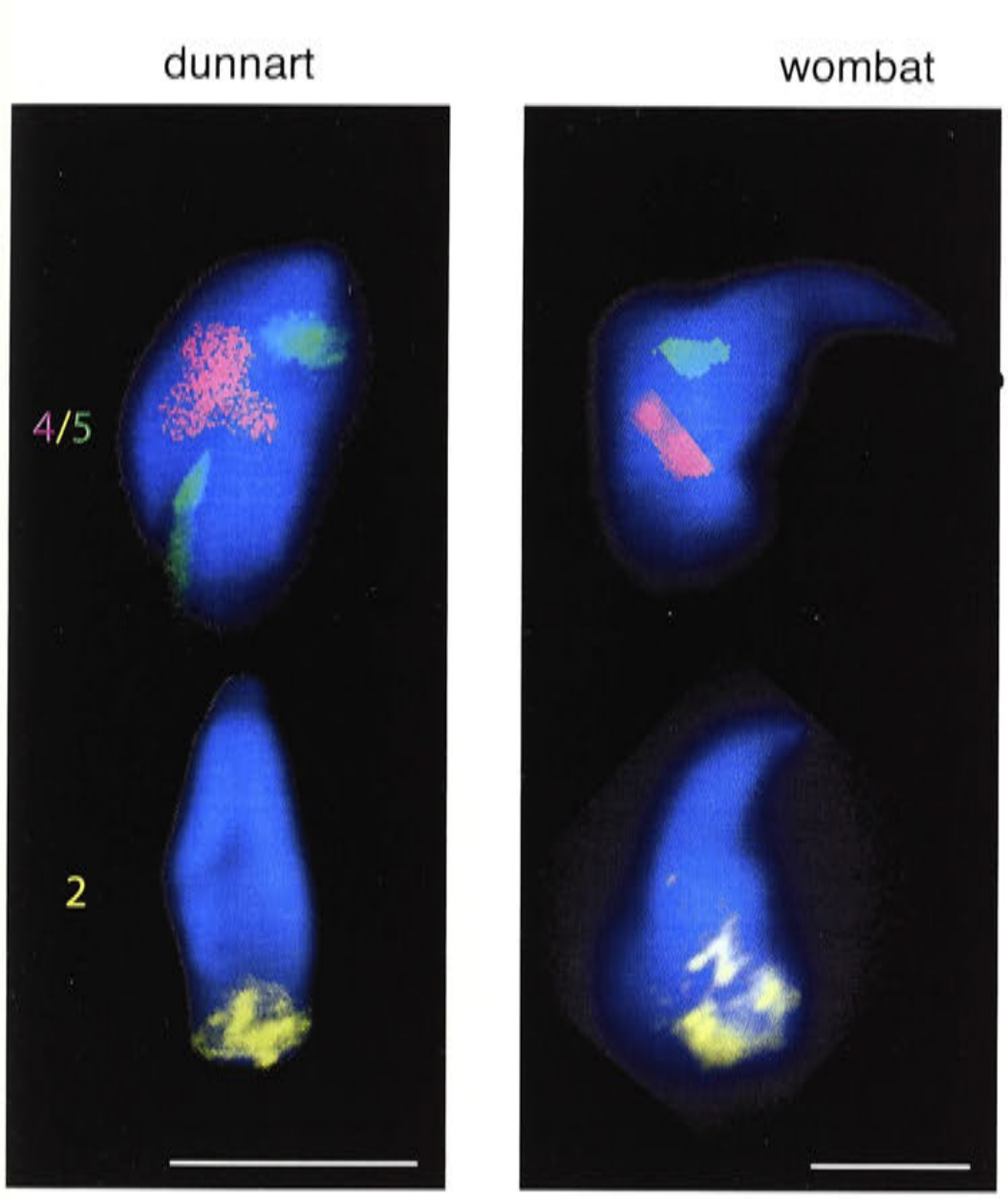
FIGURE 1



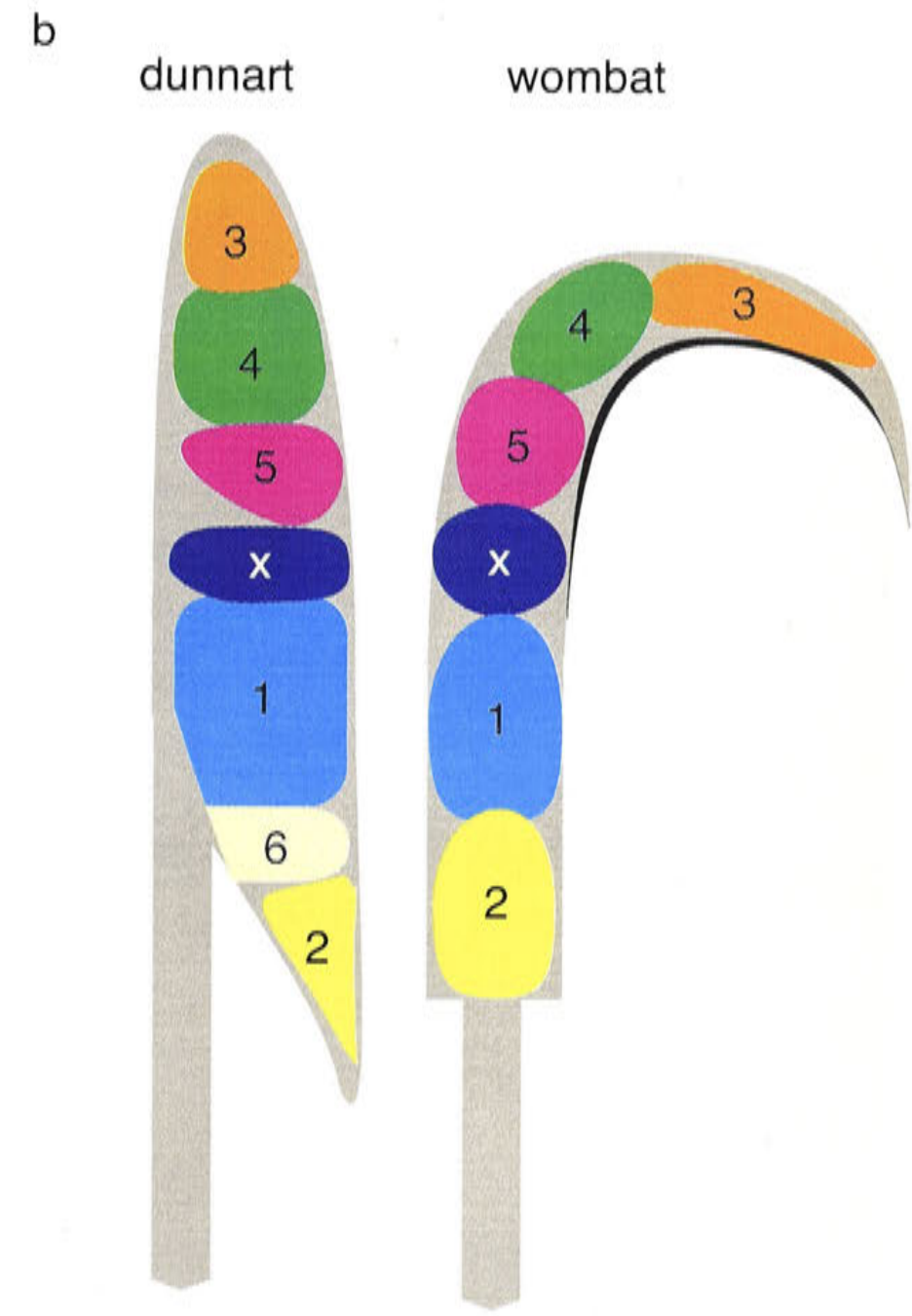


FIGURE 2

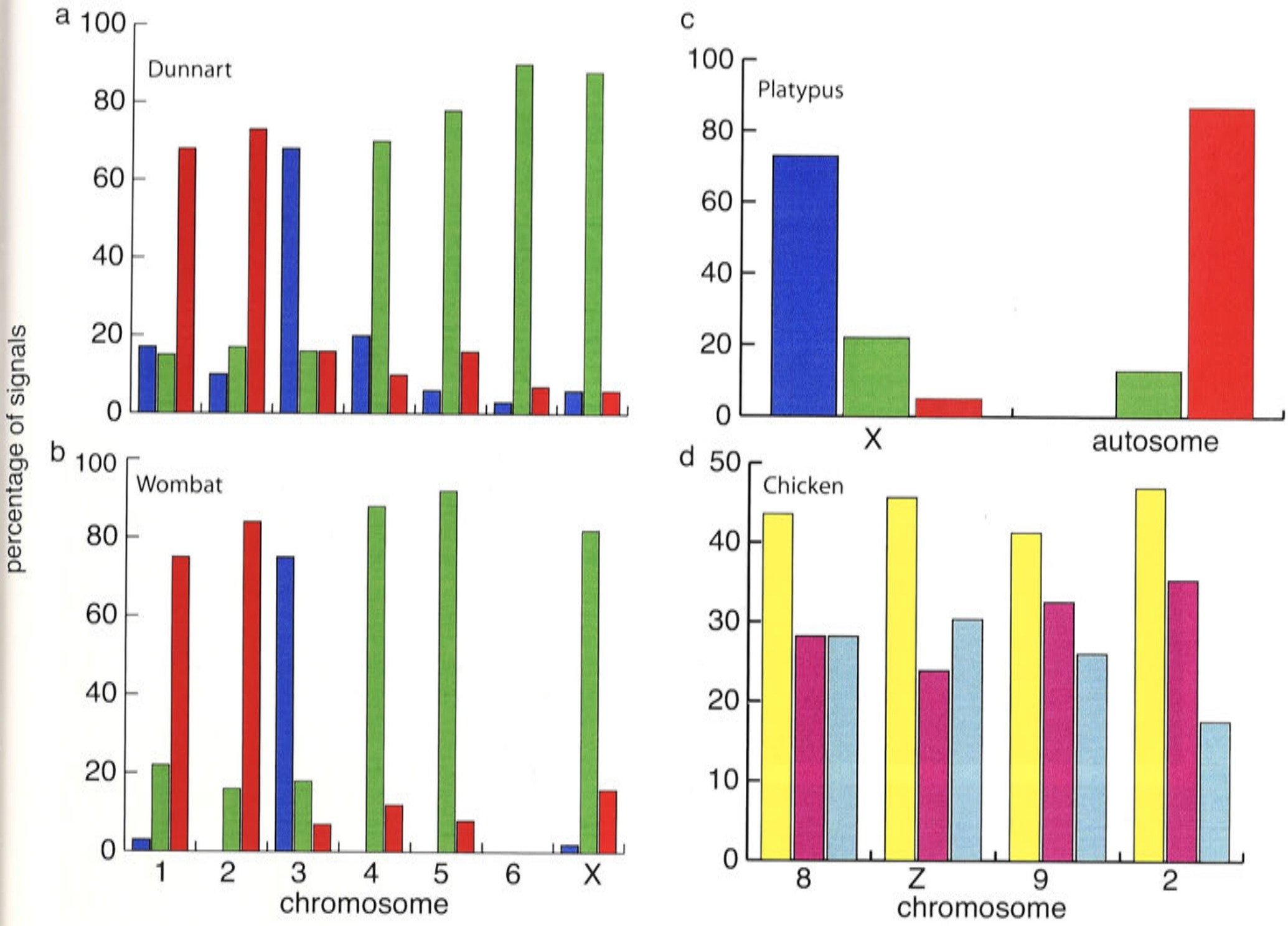

a
Dunnart
100
80
60
40
20
0
b
Wombat
100
80
60
40
20
0
1
2
3
4
5
6
X
chromosome
percentage of signals
c
Platypus
100
80
60
40
20
0
X
autosome
d
Chicken
50
40
30
20
10
0
8
Z
9
2
chromosome

Figure 3